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<p>(54) Title: ENZYMATICAL SYNTHESIS OF ssDNA</p> <p>(57) Abstract</p> <p>Methods and compositions for producing single-stranded cDNA (ss-cDNA) with a vector-based system in eukaryotic cells. The vector contains all necessary signaling instructions and enzymatic functions to allow the host cell to produce the ssDNA encoding a desired nucleic acid sequence (a "sequence of interest"). Described are the components included in the vector for synthesizing ssDNA <i>in vivo</i>. They include (1) a reverse transcriptase gene, (2) a genetic element that supplies the template for the desired ssDNA sequence of interest, and (3) a second genetic element located proximal to the genetic element encoding the sequence of interest that supplies the primer site for reverse transcription by the reverse transcriptase molecule. The vector also contains appropriate promoter(s)/enhancer(s). Also described herein is a method to construct a vector including these components.</p>			
<p>The diagram illustrates the enzymatical synthesis of ssDNA. It starts with mRNA (mRNA) which is converted to a primary transcript (mRNA'). This transcript is then processed by a ribozyme (Sequence PB5) to produce mature mRNA (mRNA'). The mature mRNA then binds to a primer binding site (PBS) on a reverse transcriptase (RT) molecule. The RT enzyme uses the mRNA as a template to synthesize single-stranded DNA (ssDNA). The ssDNA is then digested by RNaseH to remove the mRNA template, resulting in a final ssDNA product. The diagram also shows the presence of RSV (Reverse Transcriptase SV40) and CMV (Cytomegalovirus) genes, and an aptamer structure at the end of the ssDNA.</p>			

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ENZYMATIC SYNTHESIS OF ssDNA

The present invention relates to the production of single stranded DNA (ssDNA) in yeast, prokaryotic, and eukaryotic cells from a set of genetic elements delivered to the cell by a vector system. The ss DNA is produced in the cell with minimal vector sequences which could interfere with the intended function of the ssDNA in the cell.

So far as is known, there is no method for producing single-stranded deoxyribonucleic acid (ssDNA) species in eukaryotic cells which do not contain intervening and/or flanking vector sequences. The scientific and patent literature does include the disclosure of cDNA-producing vectors (*see* A. Ohshima, *et al.*, 89 Proc. Natl. Acad. Sci. USA 1016-1020 (1992); S. Inouye, *et al.*, 3 Current Opin. Genet. Develop. 713-718 (1993); O. Mirochnitchenko, *et al.*, 269 J. Biol. Chem. 2380-2383 (1994); J.-R. Mao, *et al.*, 270 J. Biol. Chem. 19684-19687 (1995); and U.S. Patent No. 5,436,141), but that system does not appear to have demonstrated the ability to produce ssDNA in eukaryotic cells without intervening vector sequences which can interfere with the intended function of the ssDNA product. It is, therefore, an object of the present invention to provide a DNA construct which directs the synthesis of ssDNA *in vitro* or *in vivo* with reduced or eliminated contiguous and/or intervening nucleotide vector sequences.

It is also an object of the present invention to provide a method for producing ssDNA and/or dsDNA *in vivo* for use as aptamers to which proteins bind for producing a therapeutic effect in a living organism.

It is also an object of the present invention to provide nucleic acid sequences, and a method of introducing such sequences into living cells, for producing a desired effect in a cell, tissue, or organism.

According to the present invention, there is provided a set of genetic elements for delivery into a cell comprising a nucleic acid construct comprising a sequence of interest, and a primer binding site for a reverse transcriptase located in a 3' position with respect to the sequence of interest.

The set of genetic elements of the present invention provides an efficient system for directing the synthesis of a stable, single-stranded nucleic acid sequence, both *in vivo*

and *in vitro*. The single-stranded nucleic acid sequence may be used to provide a desired effect in a cell, tissue or organism. Because production of the single-stranded nucleic acid sequence of interest takes place within the cell, prior art problems arising from delivery of the single-stranded nucleic acid sequence to the cell are overcome, or at least alleviated.

5 Because of the arrangement of the nucleic acid construct, with the primer binding site in a position which is 3' to the sequence of interest, there is no limit to the size or type of sequence of interest that may be produced using the nucleic acid construct of the present invention, and the construct may be easily incorporated into a vector for delivery by any desired route to a target cell.

10 Reverse transcription may be carried out by a reverse transcriptase which is endogenous to the cell (e.g. in the case of infection by human immunodeficiency virus or simian immunodeficiency virus) or the set of genetic elements may, preferably, further comprise a reverse transcriptase gene.

15 In the case that the set of genetic elements comprises a reverse transcriptase gene, the reverse transcriptase gene is, preferably, polycistrionically transcribable with the sequence of interest and primer binding site.

20 Preferably, the reverse transcriptase gene is located on the same nucleic acid construct as the sequence of interest and primer binding site and, more preferably, the reverse transcriptase gene is located in a 5' position with respect to said sequence of interest and 3' primer binding site.

The reverse transcriptase gene may encode reverse transcriptase or a reverse transcriptase/RNase H polyprotein.

25 The gene encoding reverse transcriptase/RNase H polyprotein may suitably be derived from Moloney murine leukaemia virus, human immunodeficiency virus, or simian immunodeficiency virus.

Where the set of genetic elements includes a reverse transcriptase gene, the primer binding site is, preferably, specific for the reverse transcriptase encoded by the reverse transcriptase gene. Alternatively, the primer binding site is, preferably, specific for an endogenous reverse transcriptase.

30 Preferably, the primer binding site is complementary to a transfer RNA (tRNA).

Preferably, the set of genetic elements of the present invention also comprises a promoter and, optionally, an enhancer for each of said sequence of interest and/or said

reverse transcriptase gene. More preferably, the promoter and/or enhancer is a eukaryotic promoter and/or enhancer.

The promoter may be a constitutive, inducible, wide-spectrum or tissue-specific promoter.

5 Preferably, the set of genetic elements of the present invention further comprises a polyadenylation tail sequence located in a 3' position with respect to the sequence of interest and 3' primer binding site. The polyA tail provides stability of the mRNA transcript.

10 Preferably, the sequence of interest is an antisense sequence. The present invention, thus, has far reaching uses in the field of antisense therapy, particularly in treating pathological conditions by regulating gene function.

15 The sequence of interest may also be an aptamer (i.e. an oligonucleotide that binds to a non-oligonucleotide target e.g. a protein). Thus, it can, again, be seen that the present invention, has far reaching therapeutic uses.

20 Preferably, the nucleic acid construct is DNA.

Preferably, the set of genetic elements according to any one of the preceding claims is incorporated into at least one vector.

For example, the sequence of interest and 3' primer binding site may be incorporated into a first vector, with the reverse transcriptase gene incorporated into a 20 second vector.

Alternatively, the reverse transcriptase gene, sequence of interest and primer binding site may be incorporated into a single vector. In this latter case, the reverse transcriptase gene is, preferably, located in a 5' position with respect to the sequence of interest and 3' primer binding site.

25 According to a preferred embodiment of the invention, there is provided a set of genetic elements adapted for delivery into a cell comprising

- (a) a sequence of interest and a 3' primer binding site; and
- (b) a reverse transcriptase gene,

30 said sequence of interest and 3' primer binding site, and said reverse transcriptase gene being incorporated into at last one vector for delivery into the cell.

The nucleic acid constructs of the present invention are such that they may be incorporated into commercially available delivery vectors for mammalian and human

therapeutic purposes, and may be administered by any feasible route, depending on the target cell.

According to the present invention, there is also provided a vector which comprises:

- 5 (a) a primer binding site and an insertion site for a sequence of interest, the primer binding site being located in a 3' position with respect to the insertion site; and
- (b) a reverse transcriptase gene.

Preferably, the reverse transcriptase gene is located in a 5' position with respect to the
10 insertion site and 3' primer binding site.

According to another aspect of the present invention, there is provided a vector system which comprises a first vector, comprising an insertion site for a sequence of interest and a 3' primer binding site, and a second vector which comprises a reverse transcriptase gene.

15 Preferably, the vector or vector system of the present invention is a plasmid or modified viral construct.

Preferably, the reverse transcriptase gene is operably linked to an expression control sequence.

20 The vector or vector systems of the present invention may be advantageously employed to deliver antisense, sense, triplex, or any other single-stranded nucleotide sequence of interest into a cell, using known digestion and ligation techniques to splice the sequence of interest into the vector. The vector or vector system described herein provides all the necessary signalling instructions and enzymatic functions to allow a host cell to produce a single-stranded nucleic acid molecule having a desired sequence.

25 The vector or vectors systems of the present invention may also be designed to allow the primer binding site to be removed and exchanged, so that different primer binding sites can be used, depending upon the requirements of the user and the specificity of the reverse transcriptase being used.

30 Also provided by the present invention is a host cell stably transformed or transfected with a vector or vector system of the present invention, in particular, a eukaryotic cell stably transformed or transfected with a vector or vector system of the present invention. Eukaryotic cells include yeast or plant cells, or mammalian cells.

According to the present invention there is further provided a kit for producing a single stranded nucleic acid sequence, which kit comprises a vector or vector system according to the present invention, and a restriction endonuclease for the insertion site.

According to another aspect of the present invention, there is provided a kit for 5 producing a single-stranded nucleic acid sequence, which kit comprises a vector or vector system according to the present invention, a container for the vector/vector system, and instructions for use of the vector/vector system.

According to the present invention, there is further provided an *in vivo* or *in vitro* method of producing a single-stranded nucleic acid sequence of interest, which method 10 comprises the steps of introducing a nucleic acid construct into a target cell, the nucleic acid construct comprising a sequence of interest and a primer binding site located in a 3' position with respect to the sequence of interest, transcribing the nucleic acid construct into mRNA and reverse transcribing the mRNA into cDNA.

Preferably, the method further comprises the step of removing the mRNA from an 15 mRNA/cDNA heteroduplex formed by reverse transcription of the mRNA.

Reverse transcription may be carried out either by a reverse transcriptase expressed by a reverse transcriptase gene introduced into the target cell, or by a reverse transcriptase which is endogenous to the target cell (e.g. where the target cell has been infected with human immunodeficiency virus or simian immunodeficiency virus).

20 The mRNA transcript may be removed from the mRNA/cDNA heteroduplex by means of RNase H. Preferably, the RNase H is expressed from a gene encoding a reverse transcriptase/RNase H polyprotein introduced into the target cell.

Where the single-stranded nucleic acid sequence is prepared by an *in vitro* method 25 of the present invention, the method may comprise the further step of isolating the mRNA transcript, mRNA/cDNA heteroduplex and/or single stranded cDNA from the target cell.

Also provided by the present invention, are a single-stranded cDNA transcript, an inhibitory nucleic acid molecule, (e.g. an antisense sequence or an aptamer), an mRNA transcript and/or a heteroduplex molecule produced by the *in vivo* or *in vitro* method of the present invention.

30 An inhibitory nucleic acid molecule may be single-stranded DNA synthesized from the mRNA transcript, or the mRNA transcript itself, which can specifically bind to a complementary nucleic acid sequence. Such inhibitory nucleic acid molecules are

particularly useful for regulating gene function. An inhibitory nucleic acid molecule may also be an oligo-nucleotide that specifically binds to an RNA or DNA-binding protein, or an oligo-nucleotide that binds to a biomolecule, e.g. thrombin, bradykinin or PGF2 α , which does not normally bind to RNA or DNA.

5 According to the present invention there is further provided a pharmaceutical composition which comprises a set of genetic elements, a vector or vector system, or a host cell according to the present invention, together with a pharmacologically acceptable adjuvant, diluent or carrier.

According to the present invention there is also provided a set of genetic elements, 10 a vector or vector system, or a host cell according to the present invention for use in therapy, especially for use in delivering an inhibitory nucleic acid molecule to a target cell. The set of genetic elements, vector and vector systems, and host cells of the present invention are particularly useful for alleviating pathological conditions by regulating gene expression.

15 According to a further aspect of the present invention, there is provided the use of a set of genetic elements, vector or vector system, or host cell according to the present invention for the manufacture of a medicament for alleviating a pathological condition by regulating gene expression, especially for alleviating a pathological condition by delivery of an inhibitory nucleic acid molecule to a target cell. Other uses are also disclosed.

20 The sets of genetic elements, vectors, vector systems and host cells of the present invention may be used for the prophylactic or therapeutic treatment of a wide range of conditions or diseases, particularly conditions or diseases which are caused by abnormal or altered gene expression, or conditions or diseases which may be alleviated by regulating gene expression.

25 The sets of genetic elements, vectors, host cells, kits and methods of the present invention may be used to produce single-stranded nucleic acid molecules or virtually any predefined or desired nucleotide base composition in a host cell, and are adaptable and applicable to any *in vivo* or *in vitro* system.

According to a preferred embodiment, the nucleic acid construct of the present 30 invention is an artificially synthesised, recombinant, chimeric and/or heterologous product and the sequence of interest may be foreign to the host cell in which it is introduced.

Figure 1A referenced in the following description is a schematic view of a plasmid containing genetic elements encoding the sequence of interest and a primer binding site for reverse transcriptase.

Figure 1B is a schematic view of a plasmid containing a gene for reverse
5 transcriptase.

Figure 1C is a schematic view of a plasmid containing genetic elements encoding the sequence of interest, a primer binding site, and a gene for reverse transcriptase.

Figure 2 is a schematic diagram illustrating one embodiment of the method of the present invention.

10 A vector (as used herein, the term "vector" refers to a plasmid or modified viral construct, or any other suitable vehicle, used to deliver and/or manipulate nucleic acid sequences of interest) was designed to produce ssDNA *in vivo*. The vector contains all necessary signaling instructions and enzymatic functions to allow the host cell to produce the ssDNA encoding a desired sequence (a "sequence of interest"). Described are a set of
15 genetic elements adapted for delivery into a cell by incorporation into the vector for synthesizing ssDNA *in vitro or in vivo*. They include (1) a reverse transcriptase gene, (2) a genetic element that supplies the template for the desired ssDNA sequence of interest, and (3) a second genetic element located proximal to the genetic element encoding the sequence of interest that supplies the primer site for reverse transcription by the reverse
20 transcriptase molecule. The vector also contains appropriate promoter(s)/enhancer(s). Also described herein is a method to construct a vector into which these genetic elements have been incorporated.

Regarding the reverse transcriptase gene which is the first component of the cassette, the reverse transcriptase gene from the Moloney Murine Leukemia Virus
25 (MoMuLV) was used to advantage in the examples described. Many other retroviral reverse transcriptase genes may be used to advantage in the cassette of the present invention, it being preferred that the reverse transcriptase gene is regulated by an appropriate upstream promoter/enhancer such as the Cytomegalovirus (CMV) or Rouse Sarcoma Virus (RSV) promoter for expression in eukaryotic cells.

30 The reverse transcriptase gene also preferably includes a downstream polyadenylation signal sequence so that the mRNA produced from the reverse transcriptase gene includes a 3' poly(A) tail for mRNA stability. As known to those skilled

in the art, multiple poly(A) tails are available and are routinely used for production of expressed eukaryotic genes. The reverse transcriptase produced in the cell synthesizes a complementary DNA (cDNA) from the primary mRNA transcript transcribed from the template encoding the genetic element that includes the sequence of interest as described 5 below. The RNase H activity of the reverse transcriptase, along with endogenous RNase H activity within the cell, degrades the mRNA component of the heteroduplex RNA/cDNA hybrid to produce ssDNA *in vivo*.

The second component included in the cassette encodes a nucleic acid sequence that provides the template for synthesis of ssDNA in target cells. It is this element that 10 includes the sequence of interest. As is the case for the above reverse transcriptase gene, this genetic element is preferably regulated by an appropriate wide spectrum or tissue-specific promoter(s)/enhancer(s), such as the SV-40 promoter, or combination of promoter(s)/enhancer(s), located upstream of the genetic element. Those skilled in the art who have the benefit of this disclosure will also recognize that a number of tissue-specific or wide spectrum promoters/enhancers, or combinations of promoters/enhancers 15 may be used to advantage to regulate the reverse transcriptase gene and sequence of interest. Although a list of all available promoters/enhancers is not needed to exemplify the invention, the promoters/enhancers may be constitutive or inducible and may include the CMV or RSV (non-cell type specific) or GFAP (tissue specific) promoters/enhancers 20 listed here and many other viral or mammalian promoters. Representative promoters/enhancers that are appropriate for use in connection with the present invention may include, but are not limited to, HSVtk (McKnight *et al.*, 217 Science 316, 1982), human beta-globulin (Breathnach *et al.*, 50 Ann. Rev. of Biochem. 349, 1981), beta-actin (Kawamoto *et al.*, 8 Mol. Cell Biol. 267, 1988), rat growth hormone (Larsen *et al.*, 83 25 Proc. Natl. Acad. Sci. U.S.A. 8283, 1986), MMTV (Huang *et al.*, 27 Cell 245 1981), adenovirus 5 E2 (Imperiale, *et al.*, 4 Mol. Cell. Biol. 875, 1984), SV40 (Angel *et al.*, 49 Cell 729, 1987), a-2-macroglobulin (Kunz, *et al.*, 17 Nucl. Acids Res. 1121, 1989), MHC class I gene H-2kb (Blanar *et al.*, 8 EMBO J. 1139, 1989), and thyroid stimulating 30 hormone (Chatterjee *et al.*, 86 Proc. Natl. Acad. Sci. U.S.A. 9114, 1989).

For expression in eukaryotic cells, the sequence of interest is followed downstream by a genetic element encoding for a primer-binding site (PBS) for initiation of cDNA synthesis by reverse transcription. The PBS is a sequence that is complementary to a

transfer RNA (tRNA) which resides within the eukaryotic target cell. The PBS included in the presently preferred set of genetic elements described herein was taken from the actual 18 nucleotide sequence region of MoMuLV. However, any PBS that is matched to the reverse transcriptase that comprises the set of genetic elements may be utilized for this 5 purpose. Multiple copies of the sequences of interest, each with its corresponding PBS, can be incorporated into the vector for delivery to a cell in accordance with the method of the present invention if desired, for example, for use in delivering anti-sense sequences to various regions of a gene within the target cell.

The mRNA primary transcript transcribed from the genetic element acts as the 10 template used by the reverse transcriptase described above to synthesize and process the sequence of interest, which as noted above, can be any desired ssDNA. The mRNA primary transcript contains a primer binding site (PBS) downstream to the sequence of interest. The PBS is exclusively recognized by a "primer tRNA." To those skilled in the art, tRNAs are endogenous to cells. Each tRNA has the ability to recognize a unique 15 sequence (i.e., codon) on the mRNA transcript coding for an amino acid, and has the ability to covalently link to a specific amino acid (i.e., the tRNA becomes "charged" when bound to a specific amino acid). However, a "primer tRNA" when bound to the mRNA transcript PBS and not covalently linked (i.e., "uncharged") with an amino acid, may be used to initiate ssDNA synthesis by the reverse transcriptase. For example, the MoMuLV 20 reverse transcriptase used in the examples described herein, recognizes and uses an uncharged lysine tRNA that in turn recognizes and binds to its unique sequence in the PBS. Thus, each PBS incorporated into the vector must contain the unique sequence recognized by the primer tRNA, and the primer tRNA must be one that is recognized by the particular reverse transcriptase utilized.

25 It is preferred that the vector contain other specialized genetic elements to facilitate the identification of cells that carry the set of genetic elements of the present invention and/or to increase the level of expression of the sequence of interest. The specialized genetic elements include selectable marker genes so that the vector can be transformed and amplified in a prokaryotic system. For example, the most commonly used 30 selectable markers are genes that confer to the bacteria (e.g., *E. coli*) resistance to antibiotics such as ampicillin, chloramphenicol, kanamycin (neomycin), or tetracycline. It is also preferred that the vector contain specialized genetic elements for subsequent

transfection, identification and expression in a eukaryotic system. For expression in eukaryotic cells, multiple selection strategies (e.g., Chinese Hamster Ovarian: CHO) may be used that confer to the cell resistance to an antibiotic or other drug or alter the phenotype of the cell such as morphological changes, loss of contact inhibition, or 5 increased growth rate. Selectable markers used in eukaryotic systems include, but are not limited to, resistance markers for Zeocin, resistance to G418, resistance to aminoglycoside antibiotics, or phenotypic selection markers such β -gal or green fluorescence protein.

It will also be evident to those skilled in the art from this description that the linear ssDNA can be formed into an intact stem-loop ssDNA structure by the addition of 10 inverted tandem repeats flanking the sequence of interest that form the "stem" portion after duplex formation. The stem-loop structure can function similarly in many applications as the linear ssDNA form. Such a ssDNA structure may be more resistant to intracellular nucleases by retaining the "ends" of a ssDNA in double stranded form.

It will also be evident to those skilled in the art that the stem (duplex DNA) can be 15 designed to contain a predetermined sequence or sequences (i.e., "aptamers") that are recognized and bound by specific DNA-binding proteins. Among other uses, such a stem structure is used in the cell as a competitor to titer out a selected protein(s) that regulate specific gene expression. For example, a ssDNA stem-loop of the present invention in a 20 cell such that the "stem" contains a binding site for a selected transcription factor such as adenovirus E1a. Adenovirus E1a, like other oncogenes, modulates gene expression of several adenoviral and cellular genes by affecting the activity of cell-encoded transcription factors resulting in changing normal cells to transformed cells. (Jones *et al.*, Genes Dev. 2, 267-281 (1988)). The duplex structure of the stem thus functions to "bind up" the 25 factor, preventing the protein from binding a promoter and thus inhibiting the expression of a particular deleterious gene. To those skilled in the art, it will be clear that the duplex stem structure may optionally contain multiple binding sites, for example, sites which are recognized by various transcription factors that actively regulate expression of particular gene. For example, adenovirus E1a has been found to repress transcription of the collagenase gene via the phorbol ester-responsive element, a promoter element responsible 30 for the induction of transcription by 12-O-tetradecanoylphorbol 13-acetate (TPA), by a number of other mitogens, and by the *ras*, *mos*, *src*, and *trk* oncogenes. The mechanism

involves inhibition of the function of the transcription factor family AP-1. Offringa *et al.*, 62 Cell 527-538 (1990).

In another aspect which will be recognized by those skilled in the art, the present invention is used to construct complex secondary ssDNA structures in the loop portion of 5 the DNA transcript produced in accordance with the present invention. Such secondary structure is engineered to serve any of several functions. For instance, the sequence of interest optionally includes (but is not limited to) a sequence which is incorporated into the loop portion of the single-stranded cDNA transcript to form so-called "clover leaf" or "crucible" like structures such as those found in the long terminal repeats of 10 adenoassociated virus or in retrotransposons. Under correct circumstances, such structure is integrated in site-specific manner into the host genome.

Because a vector incorporating the set of genetic elements of the present invention is adaptable for incorporation into multiple commercially available delivery vectors for mammalian and human therapeutic purposes, multiple delivery routes are feasible 15 depending upon the vector chosen for a particular target cell. For example, viral vectors are presently the most frequently used means for transforming the patient's cells and introducing DNA into the genome. In an indirect method, viral vectors, carrying new genetic information, are used to infect target cells removed from the body, and these cells are then re-implanted (i.e., *ex vivo*). Direct *in vivo* gene transfer into postnatal animals has 20 been reported for formulations of DNA encapsulated in liposomes and DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Nicolau *et al.*, Proc. Natl. Acad. Sci USA 80:1068-1072 (1983); Kaneda *et al.*, Science 243:375-378 (1989); Mannino *et al.*, Biotechniques 6:682-690 (1988). Positive results have also been described 25 with calcium phosphate co-precipitated DNA (Benvenisty and Reshef, Proc. Natl. Acad. Sci USA 83:9551-9555 (1986)). Such systems include intravenous, intramuscular, and subcutaneous injection, as well as direct intra-tumoral and intra-cavitory injections. The set of genetic elements, when incorporated into the vector of choice, can also be administered through transmucosal, rectal, oral, or inhalation-type methods of delivery.

The vector incorporating the set of genetic elements of the present invention is 30 advantageously employed to deliver antisense, sense, triplex, or any other single-stranded nucleotide sequence of interest, using known digestion and ligation techniques to splice the particular sequence of interest into the vector in the presence or absence of inverted

tandem repeats. Those skilled in the art who have the benefit of this disclosure will also recognize that the above-described signals used for expression within eukaryotic cells may be modified in ways known in the art depending upon the particular sequence of interest. The most likely change is to change the promoter so as to confer advantageous expression characteristics on the sequence of interest in the system in which it is desired to express the sequence of interest. There are so many possible promoters and other signals, and they are so dependent on the particular target cell for which the sequence of interest has been selected, that it is impossible to list all the potential enhancers, inducible and constitutive promoter systems, and/or poly(A) tailing systems which may be preferred for a particular target cell and sequence of interest.

The present invention is also utilized to produce inhibitory nucleic acids for use in therapeutics *in vivo* or *in vitro*. Inhibitory nucleic acids may be ssDNA synthesized from the mRNA template or the mRNA template itself, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA--RNA, a DNA--DNA, or RNA-DNA duplex or triplex is formed. More commonly, these nucleic acids are often termed "antisense" because they are usually complementary to the sense or coding strand of the gene, but the "sense" sequence is also utilized in the cell for therapeutic purposes. For example, the identification of oligonucleotides that specifically bind to biomolecules that do not normally bind to RNA or DNA has now been demonstrated for a number of biomolecules that vary widely in size, structure and composition. These molecules include: (1) thrombin, a multifunctional regulatory protein that converts fibrinogen to fibrin in the process of clot formation; (2) bradykinin, a nonapeptide kinin involved in blood pressure regulation and implicated in hypotension; (3) PGF2.alpha., a prostaglandin or fatty acid derivative that exhibits hormonal activity. Additionally, the interaction of oligonucleotides with biomolecules whose natural biological function is primarily extracellular has now been demonstrated. U.S. Pat. No. 5,840,867. The term "inhibitory nucleic acids" as used herein, therefore, refers to both "sense" and "antisense" nucleic acids.

By binding to the target nucleic acid, an inhibitory nucleic acid inhibits the function of the target nucleic acid. This inhibitory effect results from, for example, blocking DNA transcription, processing or poly(A) addition to mRNA, DNA replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradation.

Inhibitory nucleic acid methods therefore encompass a number of different approaches to altering expression of genes. An example of an antiherpes virus inhibitory nucleic acid is ISIS 2922 (ISIS Pharmaceuticals, Carlsbad, CA) which has activity against CMV (*see* Biotechnology News 14:5). These different types of inhibitory nucleic acid technologies
5 are described in Helene, C. and Toulme, J. (1990) Biochim. Biophys. Acta. 1049:99-125, which is referred to hereinafter as "Helene and Toulme."

In brief, inhibitory nucleic acid therapy approaches can be classified into (1) those that target DNA sequences, (2) those that target RNA sequences (including pre-mRNA and mRNA), (3) those that target proteins (sense strand approaches), and (4) those that
10 cause cleavage or chemical modification of the target nucleic acids. The first approach contemplates several categories. Nucleic acids are designed to bind to the major groove of the duplex DNA to form a triple helical or "triplex" structure. Alternatively, inhibitory nucleic acids are designed to bind to regions of single stranded DNA resulting from the opening of the duplex DNA during replication or transcription. More commonly,
15 inhibitory nucleic acids are designed to bind to mRNA or mRNA precursors. Inhibitory nucleic acids are used to prevent maturation of pre-mRNA. Inhibitory nucleic acids may be designed to interfere with RNA processing, splicing or translation. In the second approach, the inhibitory nucleic acids are targeted to mRNA. In this approach, the inhibitory nucleic acids are designed to specifically block translation of the encoded
20 protein. Using this second approach, the inhibitory nucleic acid can be used to selectively suppress certain cellular functions by inhibition of translation of mRNA encoding critical proteins. For example, an inhibitory nucleic acid complementary to regions of c-myc mRNA inhibits c-myc protein expression in a human promyelocytic leukemia cell line, HL60, which overexpresses the c-myc proto-oncogene. See Wickstrom E. L., *et al.*
25 (1988) PNAS 85:1028-1032 and Harel-Bellan, A., *et al.* (1988) Exp. Med. 168:2309-2318. As described in Helene and Toulme, inhibitory nucleic acids targeting mRNA have been shown to work by several different mechanisms to inhibit translation of the encoded protein(s).

The inhibitory nucleic acids introduced into the cell can also utilize the third
30 approach of designing the "sense" strand of the gene or mRNA to trap or compete for the enzymes or binding proteins involved in mRNA translation, as described in Helene and Toulme. Lastly, the inhibitory nucleic acids is used to induce chemical inactivation or

cleavage of the target genes or mRNA. Chemical inactivation occurs by the induction of crosslinks between the inhibitory nucleic acid and the target nucleic acid within the cell.

In another embodiment, the present invention takes the form of a kit comprised of a plasmid having the above-described reverse transcriptase gene cloned therein as well as a 5 multiple cloning site (MCS) into which the user of the kit inserts a particular sequence of interest, which may or may not include the above-described inverted tandem repeats in accordance with the user's intended result. The MCS is upstream from the genetic element encoding the primer binding site. The resulting plasmid is then purified from the cell culture in which it is maintained, lyophilized or otherwise preserved for packaging and 10 shipping to the user. The kit preferably also includes the restriction endonuclease(s) for the MCS into which the sequence of interest is to be cloned, the ligases and other enzymes for inserting the sequence of interest into the plasmid, and a map of the plasmid, along with suitable reaction buffers.

Except where otherwise indicated, standard techniques are described by Seabrook, 15 *et al.* (1989) (J. Seabrook, *et al.*, Molecular Cloning: A Laboratory Manual (2nd Ed.), Cold Spring Harbor Press (1989), hereinafter referred to as "Maniatis, *et al.* (1989)") were utilized in the examples set out below. Several experimental designs are presented to illustrate the method of producing ssDNA *in vivo*.

EXAMPLES

20 The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

Materials

The plasmid pcDNA3.1/Zeo⁺ was purchased from Invitrogen Corp. (San Diego, CA) and plasmid PBK-RSV from Statagene (La Jolla, CA). Oligodeoxyribonucleotides 25 (ODN) were synthesized by Midland Certified Reagent Co. (Midland, TX). Polymerase chain reactions (PCR) were carried out using Taq DNA polymerase purchased from Boehringer Mannheim Corp. (Indianapolis, IN) in a Robo-gradient thermal cycler (Stratagene (La Jolla, CA). Restriction endonucleases and T4 DNA ligase were obtained from Boehringer Mannheim Corp. (Indianapolis, IN). The ODNs used are listed in the 30 attached Sequence Listing.

Example 1

In vivo Synthesis of ssDNA in Eukaryotic Cells

The following *in vivo* experiments were designed to determine whether ssDNA could be produced in intact cells. To control expression of the genetic elements cloned 5 into the plasmid in these host cells, the plasmid utilized included the RSV promoter. However, those skilled in the art who have the benefit of this disclosure will recognize that any of the eukaryotic promoters listed above can be used for this purpose.

Plasmid Constructs. The cloning vector pssXB and the plasmids containing the sequences to be expressed as single-stranded DNA were constructed from a common 10 intermediate construct. The host strain for these manipulations was XL1-Blue MRF' (Stratagene, La Jolla, CA).

In the first cloning stage, to obtain the common intermediate, the vector pcDNA3.1Zeo⁺ (Invitrogen, San Diego, CA) was digested with the restriction enzymes *Nhe* I and *Apa* I. The double-stranded oligodeoxyribonucleotide having compatible *Nhe* I 15 and *Apa* I ends, which is formed by annealing the synthetic, single-stranded oligodeoxynucleotides ODN-PMMV(+) and ODN-PMMV(-) (see Table I), was ligated into the digested pcDNA3.1Zeo⁺ to give pcPMMV. This insert contains the Moloney Murine leukemia virus (MoMuLV) reverse transcriptase promoter region. It also contains two *Not* I sites, unique in pcPMMV. In this construct and in the plasmids deriving from 20 this construct, the strands designated (+) are positioned to be transcribed into RNA from the cytomegalovirus (CMV) promoter of pcDNA3.1/Zeo(+).

The plasmid pssDNA-Express-A (pssXA), containing genes for MoMuLV reverse transcriptase, was constructed from the vector pBK-RSV (Stratagene, La Jolla, CA), also using XL-1 Blue MRF' as the host strain. A mouse cell line expressing MoMuLV was 25 obtained from the American Type Culture Collection (ATCC #CRL-1858). Virus RNA was isolated and reverse transcribed from ODN-RT (-) (Table I). The reverse transcript was then PCR amplified according to the manufacturer's instructions using a kit from Promega (Madison, WI), primers ODN-RT (+) and ODN-RT (-), and digested with *Sac* I and *Hind* III (sites for these restriction endonucleases are present in the 5' and 3' primers, respectively). The 2.4 kb product obtained includes the sequence of the MoMuLV 30 genome between positions 2546 and 4908. The mature virus reverse transcriptase peptide is encoded by the sequence between positions 2337 and 4349 (Petropoulos, C.J.

Retroviral taxonomy, protein structure, sequences and genetic maps. In: Retroviruses, 757, Appendix 2, Coffin, J.M. (Ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA 1997), but peptides truncated at the amino terminus retain full activity (Sun, *et al.* (1998)).

5 The pBRK-RSV vector was digested with *Xba* I and *Nhe* I, which removes the *lac* promoter region. The *Nhe* I end was converted to a *Sac* I end using the linker formed by annealed oligodeoxynucleotides ODN-N>S (+) and ODN-N>S (-). The reverse transcriptase amplimers were ligated through the *Hind* III sites and this construct was subsequently ligated between the *Sac* I and *Xba* I sites of pBK-RSV to give
10 pBK-RSV-RT.

Those skilled in the art will recognize that the set of genetic elements comprising the present invention are also expressed from a single plasmid made by a fusion of, for instance, the pc3.1DNA/Zeo-derived plasmids and the pBK-RSV-derived plasmids such that fused plasmids encode the ss-cDNA-encoding genetic element, the Mo-MuLV-RT
15 gene, and the PBS. pBK-RSV-RT/MboL is digested with NsiI to release a 5.3kb fragment containing the Mo-MuLV-RT gene with an intervening his-pro linker and associated regulatory elements. The 5.3kb DNA fragment is ligated to a linker containing an internal EcoRI site and digested with EcoRI. The pc3.1/Zeo/N-M and the derivative plasmids containing test sequences are digested with BglII, which recognizes a unique site
20 on pc3.1DNA/Zeo in the cytomegalovirus enhancer/promoter (P CMV). The BglII ends are ligated to Seq. ID 15 and Seq. ID 16, which contain an internal EcoRI site. After digestion with EcoRI, the 5.3kb fragment is ligated to pc3.1/Zeo/N-M and derivatives to generate the plasmid.

Tissue culture studies. Stable and transient transfections are carried out by using
25 lipofectant (Boehringer Mannheim Corp.) using the manufacturer's accompanying instructions. All plasmid constructs were transfected into Cos-7, U251 and HeLa cell lines. Assays for ssDNA were performed by PCR and by dot-blot analyses 24-48 hours after transfection. Reverse transcriptase activity was assayed using the RT-PCR assay developed by Silver, et al. (Silver, J., *et al.* 21 Nucleic Acids Res. 3593-4 (1993)). The ss-
30 cDNA is isolated from cells transfected 48-72-hr earlier using triazol reagent (Gibco Life Technologies, Gaithersburg, MD). Assays for specific ss-cDNA species are carried out by both PCR based assays for internal fragment and by denatured single stranded gel

electrophoresis with subsequent nylon blotting and probing with an internal biotin-labeled probe.

The experiments described above demonstrate a method of production of ssDNA *in vivo* by multiple stepwise reactions using eukaryotic reverse transcriptase reactions and various cDNA priming reactions. Any nucleotide sequence of interest is produced by this method in a prokaryotic or eukaryotic cell. The cells were actually co-transfected with two plasmids, one plasmid carrying the genetic elements encoding the sequence of interest and primer binding site for reverse transcriptase, shown in Fig. 1A and the other carrying the gene for reverse transcriptase shown in Fig. 1B. Those skilled in the art, however, will recognize that a single plasmid including the genetic elements encoding the sequence of interest and PBS for reverse transcriptase, and the gene for reverse transcriptase also can be used for this purpose (Fig. 1C).

Example 2

Reverse Transcriptase Activity in Transformed Cells

To determine the presence of reverse transcriptase activity in extracts of cells containing the pBK-RSV-RT construct, the following assay is used. This assay relies upon reverse transcriptase activity in protein extracts of transformed cells to produce a DNA copy of the Brome Mosaic Virus RNA genome (Silver, *et al.*, 1993). The replication cycle of this virus does not involve a DNA intermediate, eliminating the possibility that an amplification product could be produced without prior reverse transcription.

Example 3

Demonstration of the presence of single-stranded DNA in Transformed Mammalian Cells.

A PCR strategy is used to detect single-stranded DNA in transformed cells. The product obtained from RNA extraction procedures, which presumably includes the single-stranded DNA is used as a template in PCR amplifications using primers specific for the expected single-stranded DNA molecule, which is not otherwise present in the cells. A band of the expected size is produced from untreated RNA/ssDNA preparations and from such preparations that were treated with RNAase A. Use of preparations treated with S1 nuclease, a highly specific, single-stranded DNA endonuclease, does not result in an amplified product.

Example 4

A method and pharmaceutical preparation
for diagnosing and treating pathological conditions
related to a dopamine receptor abnormality.

5 Abnormal activity of the dopaminergic nervous system has been implicated in a number of motor and behavioral disorders including Parkinson's disease, Huntington's disease, tardive dyskinesia, certain forms of schizophrenia and other dystonias and dyskinesias. Dysfunctions of the dopaminergic system may be caused either by a reduced or increased activity of the dopaminergic system or by the inability of the systems to be modulated by a changing external or internal environment.

10 For a patient suffering from one of the above mentioned disorders, a plasmid is constructed to include a sequence of interest that generates an antisense oligonucleotide capable of binding specifically to an expression-controlling sequence of a nucleic acid encoding the dopamine receptor. The plasmid is administered under conditions whereby 15 the plasmid enters cells expressing the dopamine receptor and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, thereby selectively controlling expression of one or more dopamine receptor subtypes, and alleviating the pathological conditions related to their expression. Efficacy is tested in accordance with the method described in U.S. 20 Patent No. 5,840,708.

Example 5

Inhibitory nucleotides to Kaposi's sarcoma-associated herpesvirus (KSHV)
virion protein 26 (VP26)

Kaposi's sarcoma-associated herpes virus (KSHV) is a new human herpes virus (HHV8) believed to cause Kaposi's sarcoma (KS). Kaposi's sarcoma is the most common neoplasm occurring in persons with acquired immunodeficiency syndrome (AIDS). Approximately 15-20% of AIDS patients develop this neoplasm which rarely occurs in immunocompetent individuals. Epidemiologic evidence suggests that AIDS-associated KS (AIDS-KS) has an infectious etiology. Gay and bisexual AIDS patients are approximately 25 twenty times more likely than hemophiliac AIDS patients to develop KS, and KS may be associated with specific sexual practices among gay men with AIDS. KS is uncommon 30 among adult AIDS patients infected through heterosexual or parenteral HIV transmission, or among pediatric AIDS patients infected through vertical HIV transmission. Agents

previously suspected of causing KS include cytomegalovirus, hepatitis B virus, human papillomavirus, Epstein-Barr virus (EBV), human herpesvirus 6, human immunodeficiency virus (HIV), and Mycoplasma penetrans. Non-infectious environmental agents, such as nitrite inhalants, also have been proposed to play a role in KS tumorigenesis. Extensive 5 investigations, however, have not demonstrated an etiologic association between any of these agents and AIDS-KS.

Virion protein 26 (VP26) is a component of the nucleocapsid structure in most herpes viruses. This structure serves as a delivery mechanism for the viral genome as it is spread from one infected cell to another. As part of the original infecting virus, it is 10 recognized as a major antigen by the immune system and can therefore be used to screen for antibodies to the herpes virus in patient sera and as a vaccine.

For an infected patient, a plasmid is constructed using the methods described above to include a sequence of interest. The sequence of interest is an isolated nucleic acid molecule which encodes KSHV virion protein 26 or antisense or triplex 15 oligonucleotide molecule as described in U.S. Patent No. 5,840,708. The plasmid is administered under conditions whereby the plasmid enters infected cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, thereby selectively controlling expression of KSHV virion protein 26, and alleviating the pathological conditions related 20 to expression.

Example 6

Inhibitory nucleotides to modulate the expression of IL-8 and/or IL-8 Receptor to control growth, metastasis and/or angiogenesis in tumors.

Interleukin-8 (IL-8, neutrophil activating protein-1, or NAP-1) is a member of C-X-C chemokine family of related cytokines having broad involvement in inflammatory responses, tissue injury, growth regulation and cellular adhesion. Cerretti, D. P., *et al.*, Molecular Characterization of Receptors for Human Interleukin-8, GRO/Melanoma Growth-Stimulatory Activity and Neutrophil Activating Peptide-2, Molecular Immunology, 30(4), 359-367 (1993); and Koch, A. E., *et al.*, In situ expression of cytokines and cellular adhesion molecules in the skin of patients with systemic sclerosis, Pathobiology, 61(5-6), 239-46 (1993). IL-8 has also been shown to have a potent 25 30

stimulatory effect on angiogenesis. See, e.g., Koch, A. E., Interleukin-8 as a Macrophage-Derived Mediator of Angiogenesis, *Science*, 258, 1798-1800 (1992).

It is known that IL-8 is produced by a variety of normal human somatic cells including monocytes/macrophages, dermal fibroblasts, vascular endothelial cells, 5 keratinocytes, and mesangeal cells. Yasumoto, K., et al., Tumor Necrosis Factor Alpha and Interferon Gamma Synergistically Induce Interleukin 8 Production in a Human Gastric Cancer Cell Line Though Acting Concurrently on AP-1 and NF-kB-like Binding Sites of the Interleukin 8 Gene, *J. of Biological Chemistry*, 267(31), 22506-11 (1992). Apparently, such cells produce IL-8 only when stressed, and not under conditions of normal growth 10 and homeostasis. Factors that induce IL-8 production include inflammation, IL-1, TNF, LPS and thrombin. It is also known that IL-8 is commonly secreted by tumor cells. Because of its effects on growth, it is suspected that IL-8 has a significant role in the metastatic spread of melanoma and other cancers.

IL-8 is a ligand for cell-membrane IL-8 Receptor, and it is thought that 15 interaction between IL-8 and IL-8 Receptor is required for IL-8 action. Two IL-8 receptor genes have been identified so far, IL-8 Receptor type A and type B. Both genes belong to the so-called seven transmembrane domain, G protein-coupled receptor family. Receptor A has been shown to be activated by IL-8, and receptor B has been shown to be activated by IL-8 as well as other cytokines belonging to C-X-C family including 20 Melanoma Growth Stimulatory Activity (MGSA).

The role and function of IL-8 Receptor B present in cancer and other tumor cells is not fully elucidated. There is, however, evidence that activation of IL-8R B (1) is involved in the mechanism of growth regulation of melanoma and tumorigenic fibroblasts; (2) is associated with transformation of lung cells by asbestos, and (3) correlates with 25 metastatic potential of melanoma.

Given the growth stimulatory effect of IL-8 on cells responsive to various tumor growth factors, it would be advantageous to provide antisense oligonucleotides which modulate expression of either IL8 or IL-8 Receptor in cancers *in vivo*. It would be particularly advantageous to provide oligonucleotides which are effective against lung 30 cancer and melanoma because each of these cancers produce their own growth factors.

There are at least two major types of lung cancer, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). SCLC comprises approximately

one-fourth of the cases, expresses neuroendocrine markers, and generally metastasizes early to lymph nodes, brain, bones, lung and liver. NSCLC comprises the majority of the remaining lung tumor types, and includes adeno-carcinoma, squamous cell carcinoma, and large cell carcinoma. NSCLC is characterized by epithelial-like growth factors and receptors, and is locally invasive.

Melanoma cells, unlike normal melanocytes, can proliferate in the absence of exogenous growth factors. This independence apparently reflects the production of growth factor and cytokines for autocrine growth stimulation, including TGF-.ANG., TGF-, platelet-derived growth factor A and B chains, basic fibroblast growth factor, IL-8, IL-6, IL-1, granulocyte macrophage colony stimulating factor, and MGSA. Guo Y, *et al.*, Inhibition of Human Melanoma Growth and Metastasis in Vivo by Anti-CD44 Monoclonal Antibody. *Cancer Res.*, 54, 1561-1565 (1994).

For a patient suffering from any of the above diseases, a plasmid is constructed using the methods described above to include a sequence of interest. The sequence of interest is an isolated nucleic acid molecule as described in U.S. Patent No. 5,849,903. To control growth, metastasis and/or angiogenesis, the plasmid is administered (e.g., inhalation or direct injection into solid tumors) under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, thereby selectively controlling expression of IL-8 receptors, and alleviating the pathological conditions related to expression.

Example 7

Antisense oligonucleotide inhibition of cytomegalovirus infection.

Cytomegaloviruses (CMVs) are ubiquitous in nature and are the most common causes of intrauterine infection. Congenital infection is common in newborns of infected mothers. In some populations, as much as 10% of children display perinatal infections. In a small percentage of newborns, the infection is virulent, involving multiple organs. Pronounced involvement of the reticuloendothelial and central nervous system is typical; and the infection is a major cause of mental retardation. Careful testing demonstrates that as many as 50% of severely, prenatally infected adults may display neuropsychiatric disease or deafness. Although extraneural organs are usually spared chronic morbidity, the virus can be detected in the kidney for years.

A plasmid is constructed using the methods described above to include a sequence of interest encoding for an inhibitory nucleotide. Oligonucleotides having a sequence of nucleotide bases specifically hybridizable with a selected sequence of a cytomegalovirus DNA or RNA are described in U.S. Patent No. 5,442,049. The plasmid is administered to the patient under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, thereby selectively controlling replication of CMV, and alleviating the pathological conditions related to CMV infection. This plasmid is used either prophylactically or therapeutically to reduce the severity of disease caused by CMV.

Example 8

Oligonucleotides specifically hybridizable with RNA or DNA
deriving from a gene corresponding to one of the open reading frames
UL5, UL8, UL9, UL20, UL27, UL29, UL30, UL42, UL52 and IE175
of herpes simplex virus type 1.

Oligonucleotides are designed to be specifically hybridizable with DNA or even more preferably, RNA from one of the species herpes simplex virus type 1 (HSV-1), herpes simplex virus type (HSV-2), cytomegalovirus, human herpes virus 6, Epstein Barr virus (EBV) or varicella zoster virus (VZV). Such oligonucleotides are conveniently and desirably presented as a pharmaceutical composition in a pharmaceutically acceptable carrier as described in U.S. Patent No. 5,514,577.

For a patient suffering from any of the above infections, a plasmid is constructed using the methods described above to include a sequence of interest. The sequence of interest is an isolated nucleic acid molecule as described in U.S. Patent No. 5,514,577. The plasmid is administered (e.g., inhalation or direct injection into solid tumors) under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, from one of the species herpes simplex virus type 1 (HSV-1), herpes simplex virus type (HSV-2), cytomegalovirus, human herpes virus 6, Epstein Barr virus (EBV) or varicella zoster virus (VZV) thereby selectively controlling virus infection, and alleviating the pathological conditions related to infection.

Example 9

Antisense oligonucleotides to proto-oncogenes, and in particular to the c-myb gene, and the use of such oligonucleotides as antineoplastic and immunosuppressive agents.

The proto-oncogene c-myb is the normal cellular homologue of the avian
5 myeloblastosis virus-transforming gene v-myb. The c-myb gene codes for a nuclear
protein expressed primarily in hematopoietic cells. It is a proto-oncogene, that is, it codes
for a protein which is required for the survival of normal, non-tumor cells. When the gene
is altered in the appropriate manner, it has the potential to become an oncogene.
Oncogenes are genes whose expression within a cell provides some function in the
10 transformation from normal to tumor cell. An example is the human c-myb gene which
has been isolated, cloned, and sequenced. Majello et al, Proc. Natl. Acad. Sci. U.S.A. 83,
9636-9640 (1986).

A plasmid is constructed using the methods described above to include a sequence
of interest encoding for an inhibitory nucleotide. Oligonucleotides having a sequence of
15 nucleotide bases specifically hybridizable with a selected sequence of the DNA or RNA as
are described in U.S. Patent No. 5,098,890. The plasmid is administered to the patient
under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide
thus acting as an antineoplastic or immunosuppressive agent.

Example 10

20 Antisense oligonucleotides Against ICAM-1 Gene Expression
in Interleukin-1 beta-Stimulated Cells.

It is has been hoped that inhibitors of ICAM-1, VCAM-1 and ELAM-1
expression would provide a novel therapeutic class of anti-inflammatory agents with
activity towards a variety of inflammatory diseases or diseases with an inflammatory
25 component such as asthma, rheumatoid arthritis, allograft rejections, inflammatory bowel
disease, various dermatological conditions, and psoriasis. In addition, inhibitors of ICAM-
1, VCAM-1, and ELAM-1 may also be effective in the treatment of colds due to
rhinovirus infection, AIDS, Kaposi's sarcoma and some cancers and their metastasis. To
date, there are no known therapeutic agents which effectively prevent the expression of
30 the cellular adhesion molecules ELAM-1, VCAM-1 and ICAM-1. The use of neutralizing
monoclonal antibodies against ICAM-1 in animal models provide evidence that such
inhibitors if identified would have therapeutic benefit for asthma; Wegner *et al.*, Science

1990, 247, 456-459, renal allografts; Cosimi et al., J. Immunol. 1990, 144, 4604-4612, and cardiac allografts; Isobe et al., Science 1992, 255, 1125-1127. The use of a soluble form of ICAM-1 molecule was also effective in preventing rhinovirus infection of cells in culture. Marlin *et al.*, 344 Nature 70-72 (1990).

5 Current agents which affect intercellular adhesion molecules include synthetic peptides, monoclonal antibodies, and soluble forms of the adhesion molecules. To date, synthetic peptides which block the interactions with VCAM-1 or ELAM-1 have not been identified. Monoclonal antibodies may prove to be useful for the treatment of acute inflammatory response due to expression of ICAM-1, VCAM-1 and ELAM-1. However,
10 with chronic treatment, the host animal develops antibodies against the monoclonal antibodies thereby limiting their usefulness. In addition, monoclonal antibodies are large proteins which may have difficulty in gaining access to the inflammatory site. Soluble forms of the cell adhesion molecules suffer from many of the same limitations as monoclonal antibodies in addition to the expense of their production and their low binding
15 affinity. Thus, there is a long felt need for molecules which effectively inhibit intercellular adhesion molecules. Antisense oligonucleotides avoid many of the pitfalls of current agents used to block the effects of ICAM-1, VCAM-1 and ELAM-1.

PCT/US90/02357 (Hession, *et al.*) discloses DNA sequences encoding Endothelial Adhesion Molecules (ELAMs), including ELAM-1 and VCAM-1 and VCAM-
20 1b. A number of uses for these DNA sequences are provided, including (1) production of monoclonal antibody preparations that are reactive for these molecules which may be used as therapeutic agents to inhibit leukocyte binding to endothelial cells; (2) production of ELAM peptides to bind to the ELAM ligand on leukocytes which, in turn, may bind to ELAM on endothelial cells, inhibiting leukocyte binding to endothelial cells; (3) use of
25 molecules binding to ELAMS (such as anti-ELAM antibodies, or markers such as the ligand or fragments of it) to detect inflammation; (4) use of ELAM and ELAM ligand DNA sequences to produce nucleic acid molecules that intervene in ELAM or ELAM ligand expression at the translational level using antisense nucleic acid and ribozymes to block translation of a specific mRNA either by masking mRNA with antisense nucleic acid
30 or cleaving it with a ribozyme.

A plasmid is constructed using the methods described above to include a sequence of interest encoding for an inhibitory nucleotide for ICAM-1, VCAM-1 or ELAM-1.

Oligonucleotides having a sequence of nucleotide bases specifically hybridizable with a selected sequence of ICAM-1, VCAM-1 or ELAM-1 DNA or RNA are described in U.S. Patent No. 5,843,738. The plasmid is administered to the patient under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide. The inhibitory 5 nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, thereby selectively controlling the expression of ICAM-1, VCAM-1 or ELAM-1, and alleviating the pathological conditions related to ICAM-1, VCAM-1 and ELAM-1 expression. This plasmid is used either prophylactically or therapeutically to reduce the severity of inflammation caused by ICAM-1, VCAM-1 and ELAM-1.

10

Example 11

Protein-Binding Oligonucleotides (Aptamers) Specifically Bind Target Molecules

The field of rational drug design using biomolecule targeting and aptamer development utilizes oligonucleotides to bind to specific proteins and thus interfere with their function. Described in U.S. Pat. No. 5,840,867, are aptamers to biomolecular targets 15 such as proteins in general, and thrombin in particular. The novel compounds and methods disclosed may be applied broadly to biotechnology diagnostics and therapeutics.

Conventional methods of detection and isolation of proteins and other molecules have employed antibodies and the like which specifically bind such substances. Recently, however, the de novo design of specifically binding oligonucleotides for non- 20 oligonucleotide targets that generally bind nucleic acids has been described. See, e.g., Blackwell, T. K., et al., *Science* (1990) 250:1104-1110; Blackwell, T. K., et al., *Science* (1990) 250:1149-1152; Tuerk, C., and Gold, L., *Science* (1990) 249:505-510; Joyce, G. F., *Gene* (1989) 82:83-87. Such oligonucleotides have been termed "aptamers" herein. Tuerk and Gold describe the use of a procedure termed "systematic evolution of ligands 25 by exponential enrichment." In this method, a pool of RNAs that are completely randomized at specific positions is subjected to selection for binding by a desired nucleic acid-binding protein which has been fixed on a nitrocellulose filter. The bound RNAs then are recovered and amplified as double-stranded DNA that is competent for subsequent in vitro transcription. The newly transcribed RNA then is recycled through this procedure to 30 enrich for oligonucleotides that have consensus sequences for binding by the cognate protein. The oligonucleotides so obtained then may be sequenced for further study. Tuerk

and Gold applied this procedure to identify RNA oligonucleotides which are bound by the RNA binding region of T4 DNA polymerase.

The identification of oligonucleotides that specifically bind to biomolecules that do not normally bind to RNA or DNA has now been demonstrated for a number of 5 biomolecules that vary widely in size, structure and composition. These molecules include: (1) thrombin, a multifunctional regulatory protein that converts fibrinogen to fibrin in the process of clot formation; (2) bradykinin, a nonapeptide kinin involved in blood pressure regulation and implicated in hypotension; (3) PGF2. α , a prostaglandin or fatty acid derivative that exhibits hormonal activity. Additionally, the interaction of oligonucleotides 10 with biomolecules whose natural biological function is primarily extracellular has now been demonstrated.

A plasmid is constructed using the methods describe above to include a sequence of interest encoding for an aptamer to thrombin. Aptamers having a sequence of nucleotide bases specifically binding to thrombin are described in U.S. Patent No. 15 5,840,867. The plasmid is administered to the patient under conditions whereby the plasmid enters cells and generates the aptamer. Alterntively, an ex vivo administration is performed where cells are removed from a patient, the plasmid is transfected into the cells, and the cells are then placed back into the patient. The aptamer binds specifically to thrombin, thereby selectively controlling the biological activity of thrombin, and alleviates 20 the pathological conditions related to thrombin's presence. This plasmid is used either prophylactically or therapeutically.

Although described with reference to the figures and specific examples set out herein, those skilled in the art will recognize that certain changes can be made to the specific elements set out herein without changing the manner in which those elements 25 function to achieve their intended respective results. For instance, the cassette described herein is described as being made up of three primary components, genetic elements which comprises a sequence of interest and primer binding site, and a reverse transcriptase gene, each of these components being provided with appropriate promoters as described herein. Those skilled in the art will recognize that, for instance, the MoMuLV reverse 30 transcriptase gene described for use as the reverse transcriptase gene of the cassette can be replaced with other reverse transcriptase genes and that promoters other than the CMV promoter may be used to advantage. All such changes and modifications which do not

depart from the spirit of the present invention are intended to fall within the scope of the following non-limiting claims.

Table I: Oligodeoxynucleotides

ODN-PMMV(+) 129 bases (#23)	5' - CTAGGTGGCGGCCGGAAGATTGGTGGCACACACACAACACAGCGCACCAATC TTCGGGCCGCCGACCCGTAGCGGGGGCTTTCATTTGGGGCTCGTCCGGAT CGGGAGACCCCTGCCAGGGCC-3'
ODN-PMMV(-) 121 bases (#24)	5' - CTGGGCAGGGGTCTCCGATCCGGACGAGCCCCAAATGAAAGACCCCGC TGACGGGTCGGCGGCCCGAAGATTGGTGCCTGTTGTGTGCGCACCAATCT TCGCGGCCGCCGAC-3'
ODN-Test (+) 57 bases (#38)	5' - GGCGGAAAGATTGGGGGCCAAAGAGTAACACTCTCAAAGGCACGGGCCAAAT CTTCC-3'
ODN-Test (-) 57 bases (#39)	5' - GGCGGAAAGATTGGGGCGCTGCCTTGAGAGTTACTCTTGGGCCAAAT CTTCC-3'
ODN-Telo (+) 92 bases (#40)	5' - GGCGGAAAGATTGGGGCGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAG GGTTAGGGTTAGGGTTAGGGCTAACCTAACCTAACCTAACCTAACCTAACCT AACCTAACCTAACCTAACGCCCCAATCTTCC-3'
ODN-Telo (-) 92 bases (#41)	5' - GGCGGAAAGATTGGGGCGCCCTAACCTAACCTAACCTAACCTAACCTAACCT AACCTAACCTAACCTAACGCCCCAATCTTCC-3'
ODN-XB(+) 51 bases	5' - GGCCTTGAAGAGCGGCCGCACTAACACCACACAGTGCGGCCGCTCTCAA- 3'
ODN-XB(-) 51 bases	5' - GGCCTTGAAGAGCGGCCGCACTGTGGTGGTTAGTGCGGCCGCTCTCAA- 3'
ODN-RT (+) 32 bases (#13)	5' - GGGATCAGGAGCTCAGATCATGGGACCAATGG-3'
ODN-RT (-) 24 bases (#12)	5' - CTTGTGCACAAGCTTGCAGGTCT-3'
ODN-N>S (+) 18 bases (#25)	5' - CTAGCGGAAGCGTAGCT-3'
ODN-N>S (-) 10 bases (#26)	5' - ACGCTTGCCG-3'
ODN-Mbo (+) 30 bases (#16)	5' - CAATTAAGGAAAGCTTGAATAATTATGTC-3'
ODN-Mbo (-) 27 bases (#33)	5' - TAATGGCCCGGGCATAGTCGGTAGGG-3'
ODN-HisPro (+) 43 bases (#36)	5' - AGCTGGATCCCCGGCTCCCCACCAACCAACACCACCCCTGCCCT-3'
ODN-HisPro (-) 42 bases (#37)	5' - AGCAGGGGCAGGGTGGTGGTGGTGGGGAGCGGGGGATCC-3'
ODN-Rep(+) 121 bases	5' - ATATCTATAATTGGCAAATCATAGCGTTATGCTGACTCAGGTGAATGC CGCGATAATTTCAGATTGCAATTTCATCAATGAATTTCAGTGATGAATTGCC AAGATTGATGTTGC-3'
ODN-Rep(-) 111 bases	5' - GACGAGATCTCCTCCAGGAATTCTCGAGAATTGGATCCCCGCTCCCCACC ACCACCAACACCACCCCTGCCCGCGATGAAAAATTATGTGAGCAACATCAATCT TGGC-3'
Seq ID 15	5' - CTAGTCGGATGCGGCGCTGCACAACAAACACAACACAGCGGGCGATCCG ATCAGCGGGGGCTTTCATTTGGGGCTCGTCCGGATCGGGAGACCCCTGCCAG GGCC-3'
Seq ID 16	5' - CTGGGGCAGGGGTCTCCGATCCGGACGAGCCCCAAATGAAAGACCCCGC TGATCGGATCGGGCGCTGTGTTGTGCGAGCGGCCGATCCGA-3'

WHAT IS CLAIMED IS:

1. A set of genetic elements for delivery into a cell comprising:
a nucleic acid construct comprising a sequence of interest; and
a primer binding site for a reverse transcriptase located in a 3' position with
respect to the sequence of interest.
2. A set of genetic elements according to Claim 1, further comprising a
reverse transcriptase gene.
3. A set of genetic elements according to Claim 2, wherein the reverse
transcriptase gene is polycistrionically transcribable with the sequence of interest and
primer binding site.
4. A set of genetic elements according to Claim 2 or 3, wherein the reverse
transcriptase gene is located on the same nucleic acid construct as the sequence of interest
and primer binding site.
5. A set of genetic elements according to any one of Claims 2 to 4, wherein
the reverse transcriptase gene is located in a 5' position with respect to said sequence of
interest and 3' primer binding site.
6. A set of genetic elements according to any one of Claims 2 to 4, wherein
the reverse transcriptase gene encodes a reverse transcriptase/RNase H polyprotein.
7. A set of genetic elements according to Claim 6, wherein the gene encoding
reverse transcriptase/RNase H polyprotein is from Moloney murine leukaemia virus,
human immunodeficiency virus, or simian immunodeficiency virus.
8. A set of genetic elements according to any one of Claims 2 to 7, wherein
the primer binding site is specific for a reverse transcriptase encoded by the reverse
transcriptase gene.
9. A set of genetic elements according to Claim 1, wherein the primer binding
site is specific for an endogenous reverse transcriptase.
10. A set of genetic elements according to any one of the preceding claims,
wherein the primer binding site is complementary to a transfer RNA (tRNA).
11. A set of genetic elements according to any one of the preceding claims,
further comprising a promoter and, optionally, an enhancer for each of said sequence of
interest and/or said reverse transcriptase gene.

12. A set of genetic elements according to Claim 11, wherein the promoter and/or enhancer is a eukaryotic promoter and/or enhancer.

13. A set of genetic elements according to Claim 11 or 12, wherein the promoter is a constitutive, inducible, wide-spectrum or tissue specific promoter.

14. A set of genetic elements according to any one of the preceding claims, further comprising a polyadenylation tail sequence located in a 3' position with respect to the sequence of interest and 3' primer binding site.

15. A set of genetic elements according to any one of the preceding claims, wherein the sequence of interest is an antisense sequence.

16. A set of genetic elements according to any one of Claims 1 to 14, wherein the sequence of interest is an aptamer.

17. A set of genetic elements according to any one of the preceding claims, wherein the nucleic acid construct is DNA.

18. A set of genetic elements according to any one of the preceding claims incorporated into at least one vector.

19. A set of genetic elements according to any one of Claims 2-17, wherein the sequence of interest and 3' primer binding site are incorporated into a first vector, and wherein the reverse transcriptase gene is incorporated into a second vector.

20. A set of genetic elements according to any one of Claims 2-17, wherein the reverse transcriptase gene, sequence of interest and primer binding site are incorporated into a single vector.

21. A set of genetic elements according to Claim 20, wherein the reverse transcriptase gene is located in a 5' position with respect to the sequence of interest and 3' primer binding site.

22. A set of genetic elements adapted for delivery into a cell comprising

- (a) a sequence of interest and a 3' primer binding site; and
- (b) a reverse transcriptase gene,

said sequence of interest and 3' primer binding site, and said reverse transcriptase gene being incorporated into at last one vector for delivery into the cell.

23. A vector which comprises:

- (a) a primer binding site and an insertion site for a sequence of interest, the primer binding site being located in a 3' position with respect to the insertion site; and
- (b) a reverse transcriptase gene.

24. A vector according to Claim 23, wherein the reverse transcriptase gene is located in a 5' position with respect to the insertion site and 3' primer binding site

25. A vector system which comprises a first vector, comprising an insertion site for a sequence of interest and a 3' primer binding site and a second vector which comprises a reverse transcriptase gene.

26. A vector or vector system according to any one of Claims 23 to 25, wherein the vector is a plasmid or modified viral construct.

27. A vector or vector system according to any one of Claims 23 to 26, wherein the reverse transcriptase gene is operably linked to an expression control sequence.

28. A host cell stably transformed or transfected with a vector or vector system according to any of Claims 23 to 27.

29. A host cell according to Claim 28, which is a eukaryotic cell.

30. A kit for producing a single-stranded nucleic acid sequence, which kit comprises a vector or vector system according to any one of Claims 23 to 27, and a restriction endonuclease for the insertion site.

31. A kit for producing a single-stranded nucleic acid sequence, which kit comprises a vector or vector system according to any one of Claims 23 to 27, a container for the vector/vector system and instructions for use of the vector/vector system.

32. An *in vivo* or *in vitro* method of producing a single-stranded nucleic acid sequence of interest, which method comprises the steps of introducing a nucleic acid construct into a target cell, the nucleic acid construct comprising a sequence of interest and a primer binding site located in a 3' position with respect to the sequence of interest, transcribing the nucleic acid construct into mRNA and reverse transcribing the mRNA into cDNA.

33. A method according to Claim 32, further comprising the step of removing the mRNA from an mRNA/cDNA heteroduplex formed by reverse transcription of the mRNA.

34. A method according to Claim 32 or 33, wherein reverse transcription is carried out by a reverse transcriptase expressed by a reverse transcriptase gene introduced into the target cell.

35. A method according to Claim 32 or 33, wherein reverse transcription is carried out by a reverse transcriptase which is endogenous to the target cell.

36. A method according to any one of Claims 33 to 35, wherein the mRNA transcript is removed from the mRNA/cDNA heteroduplex by means of RNase H.

37. A method according to Claim 36, wherein the RNase H is expressed from a gene encoding a reverse transcriptase/RNase H polyprotein introduced into the target cell.

38. A method according to any one of Claims 32 to 37, further comprising the step of isolating the mRNA transcript, mRNA/cDNA heteroduplex and/or single-stranded cDNA from the target cell.

39. A single-stranded cDNA transcript produced by the method of any one of Claims 32 to 38.

40. An inhibitory nucleic acid molecule produced by the method of any one of Claims 32 to 38.

41. An inhibitory nucleic acid molecule according to Claim 40, which is an antisense sequence or an aptamer.

42. An mRNA transcript produced by the method of any one of Claims 32 to 38.

43. A heteroduplex molecule produced by the method of any one of Claims 32 to 38.

44. A pharmaceutical composition which comprises a set of genetic elements according to any one of Claims 1 to 22, together with a pharmacologically acceptable adjuvant, diluent or carrier.

45. A pharmaceutical composition which comprises a vector or vector system according to any one of Claims 23 to 27, together with a pharmacologically acceptable adjuvant, diluent or carrier.

46. A pharmaceutical composition which comprises a host cell according to Claim 28 or 29, together with a pharmacologically acceptable adjuvant, diluent or carrier.

47. A set of genetic elements according to any one of Claims 1 to 22 for use in therapy, especially for use in delivering an inhibitory nucleic acid molecule to a target cell.

48. A vector or vector system according to any one of Claims 23 to 27 for use in therapy, especially for use in delivering an inhibitory nucleic acid molecule to a target cell.

49. A host cell according to Claim 28 or 29 for use in therapy, especially for use in delivering an inhibitory nucleic acid molecule to a target cell.

50. Use of a set of genetic elements according to any one of Claims 1 to 22, for the manufacture of a medicament for alleviating a pathological condition by regulating gene expression, especially for alleviating a pathological condition by delivery of an inhibitory nucleic acid molecule to a target cell.

51. Use of a vector or vector system according to any one of Claims 23 to 27, for the manufacture of a medicament for alleviating a pathological condition by regulating gene expression, especially for alleviating a pathological condition by delivery of an inhibitory nucleic acid molecule to a target cell.

52. Use of a host cell according to Claim 28 or 29 for the manufacture of a medicament for alleviating a pathological condition by regulating gene expression, especially for alleviating a pathological condition by delivery of an inhibitory nucleic acid molecule to a target cell.

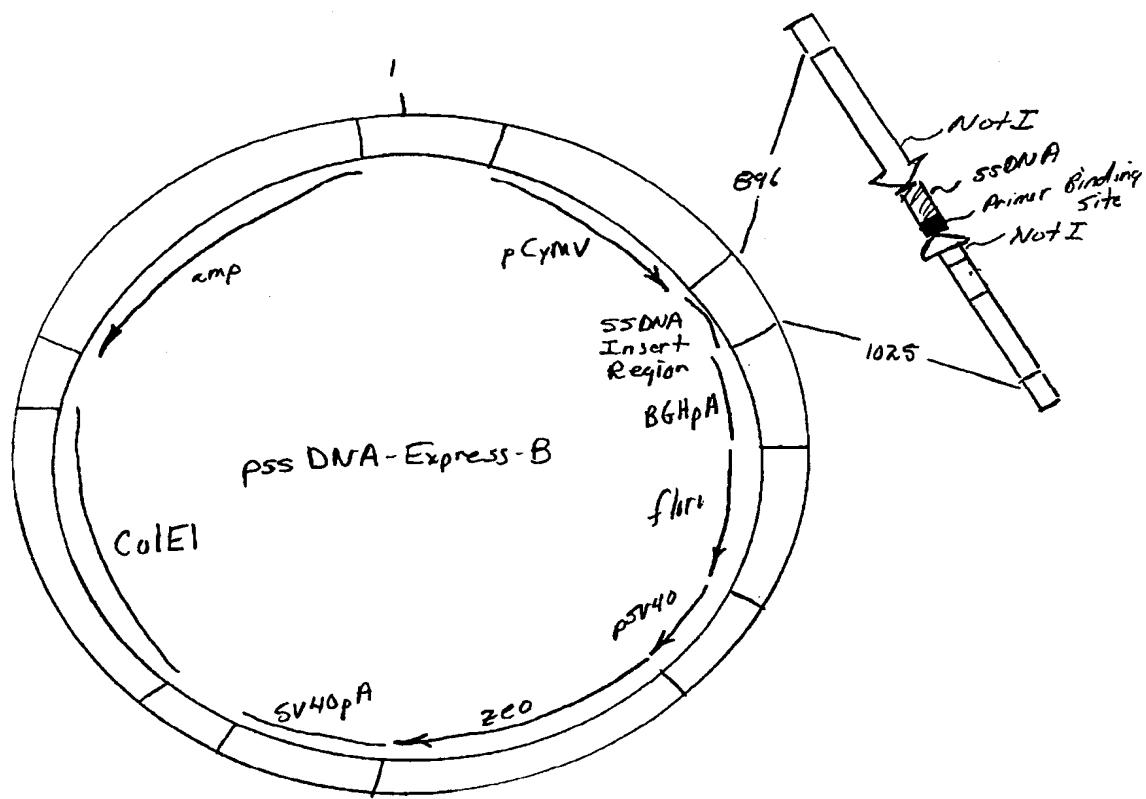


Fig 1A

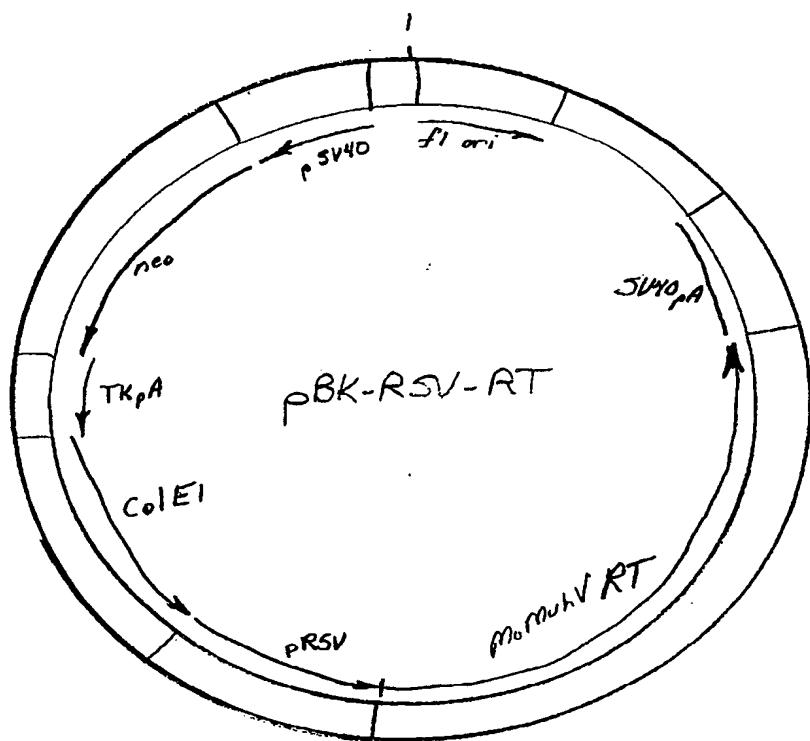


Fig. 1B

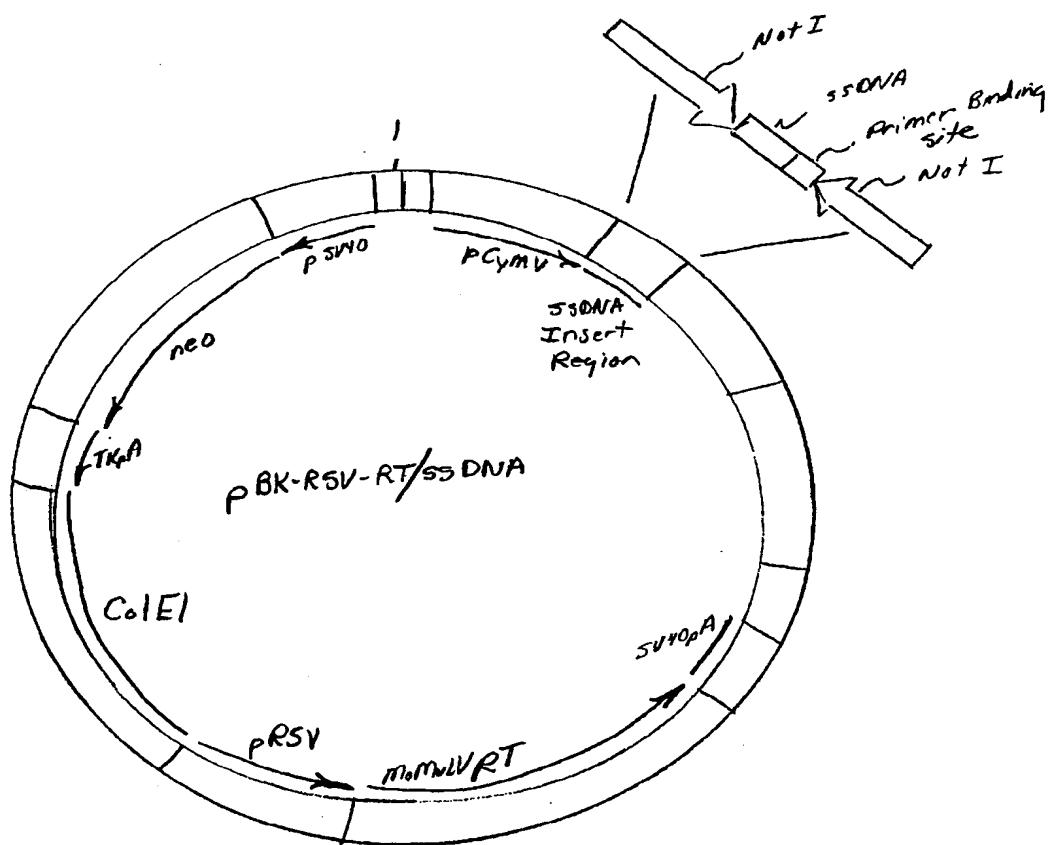


Fig 1C

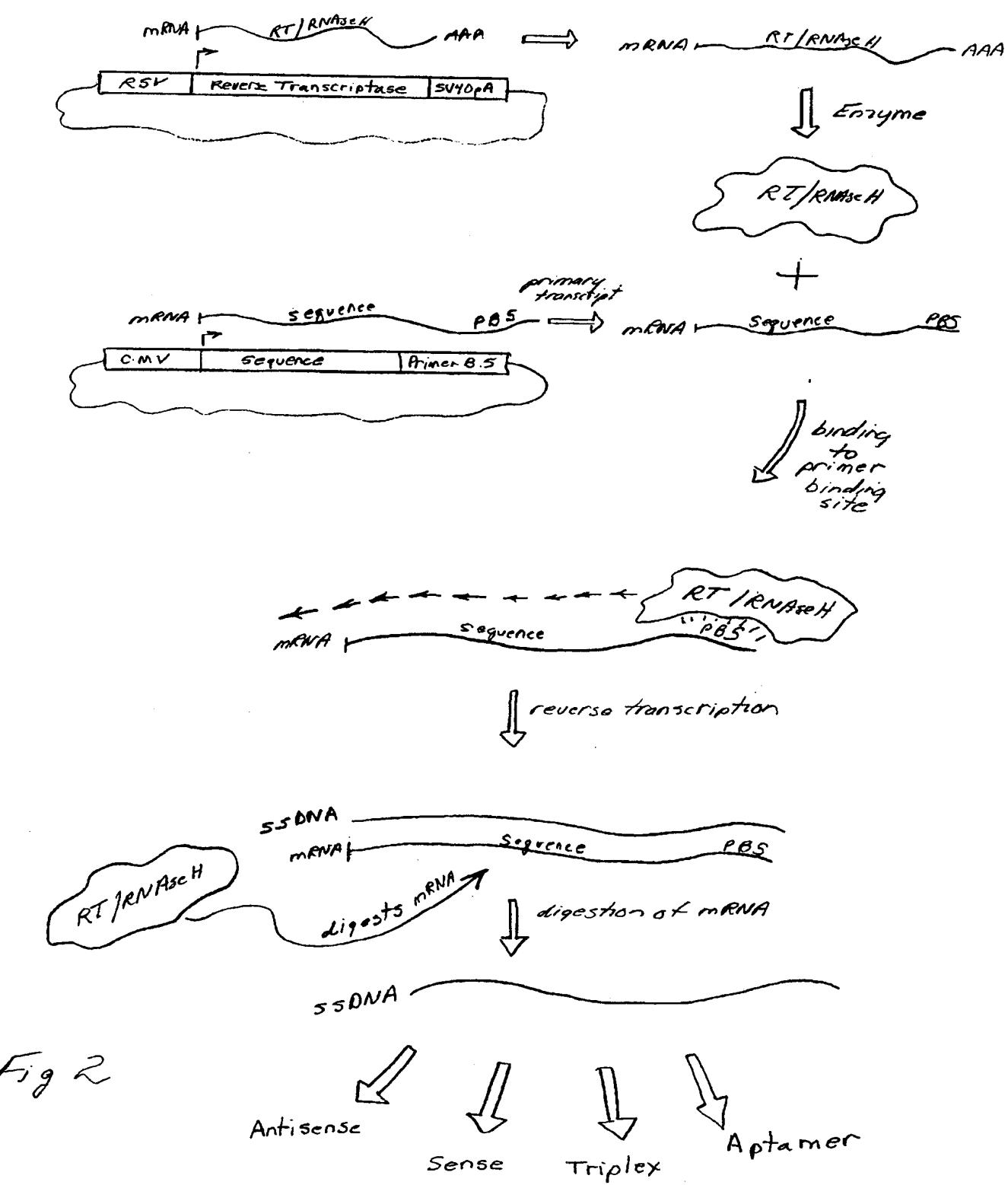


Fig 2

Antisense Sense Triplex Aptamer

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/23933

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/10 C12N15/11 C12N15/85 C12N5/10 A61K31/711
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

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IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^a	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 13689 A (MILLER JEFFREY E) 23 June 1994 (1994-06-23) the whole document ---	1,2, 6-13, 17-19, 42,43
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Further documents are listed in the continuation of box C.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	EP 0 532 380 A (UNIV MEDICINE AND DENTISTRY OF) 17 March 1993 (1993-03-17) cited in the application page 9, line 36 -page 10, column 27; claims 45-47; figure 10 ---	
A	EP 0 562 206 A (UNIV MEDICINE AND DENTISTRY OF) 29 September 1993 (1993-09-29) page 10, line 54 -page 11, line 45 ---	
A	O. MIROCHNITCHENKO ET AL.: "Production of single-stranded DNA in mammalian cells by means of a bacterial retron" J. BIOL. CHEM., vol. 269, no. 4, 28 January 1994 (1994-01-28), pages 2380-2383, XP002132577 AM. SOC. BIOCHEM. MOL.BIOL.,INC.,BALTIMORE,US cited in the application the whole document ---	
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A	OHSHIMA A ET AL: "IN-VIVO DUPLICATION OF GENETIC ELEMENTS BY THE FORMATION OF STEM-LOOP DNA WITHOUT AN RNA INTERMEDIATE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 1992, vol. 89, no. 3, 1992, pages 1016-1020, XP002132579 ISSN: 0027-8424 cited in the application the whole document ---	
A	EP 0 530 112 A (UNIV MEDICINE AND DENTISTRY OF) 3 March 1993 (1993-03-03) cited in the application claims 34,35 ---	
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INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/23933

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 94 20639 A (OHSHIMA ATUSHI ; INOUYE SUMIKO (US); INOUYE MASAYORI (US); UNIV MED) 15 September 1994 (1994-09-15) cited in the application page 56, line 3 - line 9; claims 22,23 -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int'l Application No

PCT/US 99/23933

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
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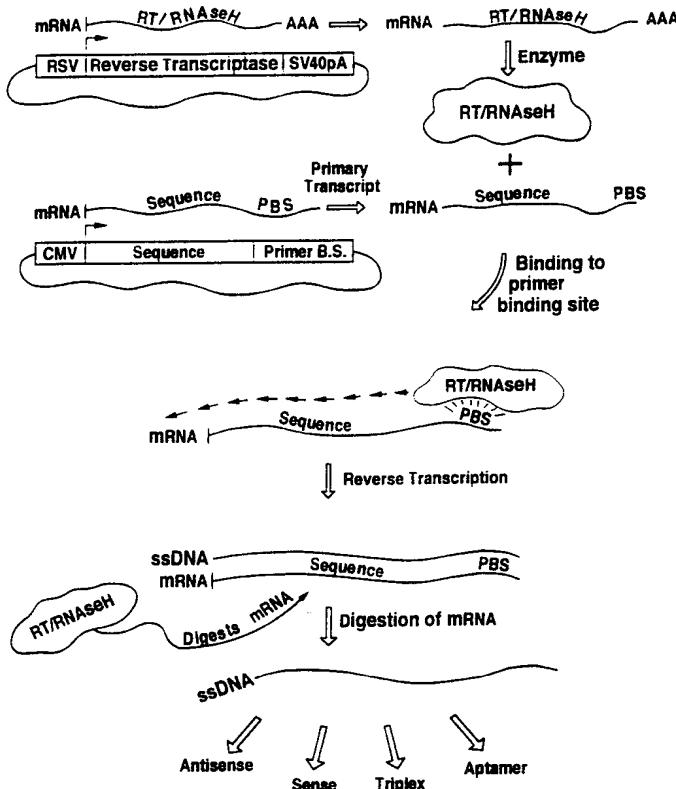
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(51) International Patent Classification ⁷ :	A1	(11) International Publication Number: WO 00/22113
C12N 15/10, 15/11, 15/85, 5/10, A61K 31/711, 48/00		(43) International Publication Date: 20 April 2000 (20.04.00)
(21) International Application Number: PCT/US99/23933		(74) Agent: WISNER, Mark, R.; Wisner & Associates, Suite 930, 2925 Briarpark, Houston, TX 77042-3728 (US).
(22) International Filing Date: 12 October 1999 (12.10.99)		
(30) Priority Data:		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).
09/169,793 9 October 1998 (09.10.98) US		
09/397,783 16 September 1999 (16.09.99) US		
(63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application		
US 09/397,783 (CIP)		
Filed on 16 September 1999 (16.09.99)		
(71) Applicants (for all designated States except US): INGENE, INC. [US/US]; 8496 W. 116th Street, Over Land Park, KS 66210 (US). CRYOGENIC SOLUTIONS, INC. [US/US]; Suite 388, 6524 San Felipe, Houston, TX 77057 (US).		
(72) Inventors; and		
(75) Inventors/Applicants (for US only): SKILLERN, Michael, J. [US/US]; 5868 A-1 Westheimer, PMB 466, Houston, TX 77057 (US). CONRAD, Charles, A. [US/US]; 8496 W. 116th Street, Over Land Park, KS 66210 (US). ELLISTON, Jonathan, F. [US/US]; 3626 Tartan Lane, Houston, TX 77025 (US).		

(54) Title: ENZYMATICAL SYNTHESIS OF ssDNA

(57) Abstract

Methods and compositions for producing single-stranded cDNA (ss-cDNA) with a vector-based system in eukaryotic cells. The vector contains all necessary signaling instructions and enzymatic functions to allow the host cell to produce the ssDNA encoding a desired nucleic acid sequence (a "sequence of interest"). Described are the components included in the vector for synthesizing ssDNA *in vivo*. They include (1) a reverse transcriptase gene, (2) a genetic element that supplies the template for the desired ssDNA sequence of interest, and (3) a second genetic element located proximal to the genetic element encoding the sequence of interest that supplies the primer site for reverse transcription by the reverse transcriptase molecule. The vector also contains appropriate promoter(s)/enhancer(s). Also described herein is a method to construct a vector including these components.



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ENZYMATIC SYNTHESIS OF ssDNA

The present invention relates to the production of single stranded DNA (ssDNA) in yeast, prokaryotic, and eukaryotic cells from a set of genetic elements delivered to the cell by a vector system. The ss DNA is produced in the cell with minimal vector sequences which could interfere with the intended function of the ssDNA in the cell.

So far as is known, there is no method for producing single-stranded deoxyribonucleic acid (ssDNA) species in eukaryotic cells which do not contain intervening and/or flanking vector sequences. The scientific and patent literature does include the disclosure of cDNA-producing vectors (*see A. Ohshima, et al.*, 89 Proc. Natl. Acad. Sci. USA 1016-1020 (1992); *S. Inouye, et al.*, 3 Current Opin. Genet. Develop. 713-718 (1993); *O. Mirochnitchenko, et al.*, 10 269 J. Biol. Chem. 2380-2383 (1994); *J.-R. Mao, et al.*, 270 J. Biol. Chem. 19684-19687 (1995); and U.S. Patent No. 5,436,141), but that system does not appear to have demonstrated the ability to produce ssDNA in eukaryotic cells without intervening vector sequences which can interfere with the intended function of the ssDNA product. It is, therefore, an object of the present invention to provide a DNA construct which directs the synthesis of ssDNA *in vitro* or 15 *in vivo* with reduced or eliminated contiguous and/or intervening nucleotide vector sequences.

It is also an object of the present invention to provide a method for producing ssDNA and/or dsDNA *in vivo* for use as aptamers to which proteins bind for producing a therapeutic effect in a living organism.

It is also an object of the present invention to provide nucleic acid sequences, and a 20 method of introducing such sequences into living cells, for producing a desired effect in a cell, tissue, or organism.

According to the present invention, there is provided a set of genetic elements for delivery into a cell comprising a nucleic acid construct comprising a sequence of interest, and a primer binding site for a reverse transcriptase located in a 3' position with respect to the 25 sequence of interest.

The set of genetic elements of the present invention provides an efficient system for directing the synthesis of a stable, single-stranded nucleic acid sequence, both *in vivo* and *in vitro*. The single-stranded nucleic acid sequence may be used to provide a desired effect in a cell, tissue or organism. Because production of the single-stranded nucleic acid sequence of

interest takes place within the cell, prior art problems arising from delivery of the single-stranded nucleic acid sequence to the cell are overcome, or at least alleviated.

Because of the arrangement of the nucleic acid construct, with the primer binding site in a position which is 3' to the sequence of interest, there is no limit to the size or type of sequence of interest that may be produced using the nucleic acid construct of the present invention, and the construct may be easily incorporated into a vector for delivery by any desired route to a target cell.

Reverse transcription may be carried out by a reverse transcriptase which is endogenous to the cell (e.g. in the case of infection by human immunodeficiency virus or simian immunodeficiency virus) or the set of genetic elements may, preferably, further comprise a reverse transcriptase gene.

In the case that the set of genetic elements comprises a reverse transcriptase gene, the reverse transcriptase gene is, preferably, polycistrionically transcribable with the sequence of interest and primer binding site.

Preferably, the reverse transcriptase gene is located on the same nucleic acid construct as the sequence of interest and primer binding site and, more preferably, the reverse transcriptase gene is located in a 5' position with respect to said sequence of interest and 3' primer binding site.

The reverse transcriptase gene may encode reverse transcriptase or a reverse transcriptase/RNase H polyprotein.

The gene encoding reverse transcriptase/RNase H polyprotein may suitably be derived from Moloney murine leukaemia virus, human immunodeficiency virus, or simian immunodeficiency virus.

Where the set of genetic elements includes a reverse transcriptase gene, the primer binding site is, preferably, specific for the reverse transcriptase encoded by the reverse transcriptase gene. Alternatively, the primer binding site is, preferably, specific for an endogenous reverse transcriptase.

Preferably, the primer binding site is complementary to a transfer RNA (tRNA).

Preferably, the set of genetic elements of the present invention also comprises a promoter and, optionally, an enhancer for each of said sequence of interest and/or said reverse

transcriptase gene. More preferably, the promoter and/or enhancer is a eukaryotic promoter and/or enhancer.

The promoter may be a constitutive, inducible, wide-spectrum or tissue-specific promoter.

5 Preferably, the set of genetic elements of the present invention further comprises a polyadenylation tail sequence located in a 3' position with respect to the sequence of interest and 3' primer binding site. The polyA tail provides stability of the mRNA transcript.

Preferably, the sequence of interest is an antisense sequence. The present invention, thus, has far reaching uses in the field of antisense therapy, particularly in treating pathological 10 conditions by regulating gene function.

The sequence of interest may also be an aptamer (i.e. an oligonucleotide that binds to a non-oligonucleotide target e.g. a protein). Thus, it can, again, be seen that the present invention, has far reaching therapeutic uses.

Preferably, the nucleic acid construct is DNA.

15 Preferably, the set of genetic elements according to any one of the preceding claims is incorporated into at least one vector.

For example, the sequence of interest and 3' primer binding site may be incorporated into a first vector, with the reverse transcriptase gene incorporated into a second vector.

20 Alternatively, the reverse transcriptase gene, sequence of interest and primer binding site may be incorporated into a single vector. In this latter case, the reverse transcriptase gene is, preferably, located in a 5' position with respect to the sequence of interest and 3' primer binding site.

According to a preferred embodiment of the invention, there is provided a set of genetic elements adapted for delivery into a cell comprising

25 (a) a sequence of interest and a 3' primer binding site; and
(b) a reverse transcriptase gene,

said sequence of interest and 3' primer binding site, and said reverse transcriptase gene being incorporated into at last one vector for delivery into the cell.

30 The nucleic acid constructs of the present invention are such that they may be incorporated into commercially available delivery vectors for mammalian and human

therapeutic purposes, and may be administered by any feasible route, depending on the target cell.

According to the present invention, there is also provided a vector which comprises:

5 (a) a primer binding site and an insertion site for a sequence of interest, the primer binding site being located in a 3' position with respect to the insertion site; and
 (b) a reverse transcriptase gene.

Preferably, the reverse transcriptase gene is located in a 5' position with respect to the insertion site and 3' primer binding site.

According to another aspect of the present invention, there is provided a vector system 10 which comprises a first vector, comprising an insertion site for a sequence of interest and a 3' primer binding site, and a second vector which comprises a reverse transcriptase gene.

Preferably, the vector or vector system of the present invention is a plasmid or modified viral construct.

Preferably, the reverse transcriptase gene is operably linked to an expression control 15 sequence.

The vector or vector systems of the present invention may be advantageously employed to deliver antisense, sense, triplex, or any other single-stranded nucleotide sequence of interest into a cell, using known digestion and ligation techniques to splice the sequence of interest into the vector. The vector or vector system described herein provides all the 20 necessary signalling instructions and enzymatic functions to allow a host cell to produce a single-stranded nucleic acid molecule having a desired sequence.

The vector or vectors systems of the present invention may also be designed to allow the primer binding site to be removed and exchanged, so that different primer binding sites can be used, depending upon the requirements of the user and the specificity of the reverse 25 transcriptase being used.

Also provided by the present invention is a host cell stably transformed or transfected with a vector or vector system of the present invention, in particular, a eukaryotic cell stably transformed or transfected with a vector or vector system of the present invention. Eukaryotic cells include yeast or plant cells, or mammalian cells.

According to the present invention there is further provided a kit for producing a single stranded nucleic acid sequence, which kit comprises a vector or vector system according to the present invention, and a restriction endonuclease for the insertion site.

According to another aspect of the present invention, there is provided a kit for 5 producing a single-stranded nucleic acid sequence, which kit comprises a vector or vector system according to the present invention, a container for the vector/vector system, and instructions for use of the vector/vector system.

According to the present invention, there is further provided an *in vivo* or *in vitro* method of producing a single-stranded nucleic acid sequence of interest, which method 10 comprises the steps of introducing a nucleic acid construct into a target cell, the nucleic acid construct comprising a sequence of interest and a primer binding site located in a 3' position with respect to the sequence of interest, transcribing the nucleic acid construct into mRNA and reverse transcribing the mRNA into cDNA.

Preferably, the method further comprises the step of removing the mRNA from an 15 mRNA/cDNA heteroduplex formed by reverse transcription of the mRNA.

Reverse transcription may be carried out either by a reverse transcriptase expressed by a reverse transcriptase gene introduced into the target cell, or by a reverse transcriptase which is endogenous to the target cell (e.g. where the target cell has been infected with human immunodeficiency virus or simian immunodeficiency virus).

20 The mRNA transcript may be removed from the mRNA/cDNA heteroduplex by means of RNase H. Preferably, the RNase H is expressed from a gene encoding a reverse transcriptase/RNase H polyprotein introduced into the target cell.

Where the single-stranded nucleic acid sequence is prepared by an *in vitro* method of 25 the present invention, the method may comprise the further step of isolating the mRNA transcript, mRNA/cDNA heteroduplex and/or single stranded cDNA from the target cell.

Also provided by the present invention, are a single-stranded cDNA transcript, an inhibitory nucleic acid molecule, (e.g. an antisense sequence or an aptamer), an mRNA transcript and/or a heteroduplex molecule produced by the *in vivo* or *in vitro* method of the present invention.

30 An inhibitory nucleic acid molecule may be single-stranded DNA synthesized from the mRNA transcript, or the mRNA transcript itself, which can specifically bind to a

complementary nucleic acid sequence. Such inhibitory nucleic acid molecules are particularly useful for regulating gene function. An inhibitory nucleic acid molecule may also be an oligonucleotide that specifically binds to an RNA or DNA-binding protein, or an oligo-nucleotide that binds to a biomolecule, e.g. thrombin, bradykinin or PGF 2α , which does not normally bind 5 to RNA or DNA.

According to the present invention there is further provided a pharmaceutical composition which comprises a set of genetic elements, a vector or vector system, or a host cell according to the present invention, together with a pharmacologically acceptable adjuvant, diluent or carrier.

10 According to the present invention there is also provided a set of genetic elements, a vector or vector system, or a host cell according to the present invention for use in therapy, especially for use in delivering an inhibitory nucleic acid molecule to a target cell. The set of genetic elements, vector and vector systems, and host cells of the present invention are particularly useful for alleviating pathological conditions by regulating gene expression.

15 According to a further aspect of the present invention, there is provided the use of a set of genetic elements, vector or vector system, or host cell according to the present invention for the manufacture of a medicament for alleviating a pathological condition by regulating gene expression, especially for alleviating a pathological condition by delivery of an inhibitory nucleic acid molecule to a target cell. Other uses are also disclosed.

20 The sets of genetic elements, vectors, vector systems and host cells of the present invention may be used for the prophylactic or therapeutic treatment of a wide range of conditions or diseases, particularly conditions or diseases which are caused by abnormal or altered gene expression, or conditions or diseases which may be alleviated by regulating gene expression.

25 The sets of genetic elements, vectors, host cells, kits and methods of the present invention may be used to produce single-stranded nucleic acid molecules or virtually any predefined or desired nucleotide base composition in a host cell, and are adaptable and applicable to any *in vivo* or *in vitro* system.

According to a preferred embodiment, the nucleic acid construct of the present 30 invention is an artificially synthesised, recombinant, chimeric and/or heterologous product and the sequence of interest may be foreign to the host cell in which it is introduced.

Figure 1A referenced in the following description is a schematic view of a plasmid containing genetic elements encoding the sequence of interest and a primer binding site for reverse transcriptase.

Figure 1B is a schematic view of a plasmid containing a gene for reverse transcriptase.

5 Figure 1C is a schematic view of a plasmid containing genetic elements encoding the sequence of interest, a primer binding site, and a gene for reverse transcriptase.

Figure 2 is a schematic diagram illustrating one embodiment of the method of the present invention.

A vector (as used herein, the term "vector" refers to a plasmid or modified viral construct, or any other suitable vehicle, used to deliver and/or manipulate nucleic acid sequences of interest) was designed to produce ssDNA *in vivo*. The vector contains all necessary signaling instructions and enzymatic functions to allow the host cell to produce the ssDNA encoding a desired sequence (a "sequence of interest"). Described are a set of genetic elements adapted for delivery into a cell by incorporation into the vector for synthesizing ssDNA *in vitro or in vivo*. They include (1) a reverse transcriptase gene, (2) a genetic element that supplies the template for the desired ssDNA sequence of interest, and (3) a second genetic element located proximal to the genetic element encoding the sequence of interest that supplies the primer site for reverse transcription by the reverse transcriptase molecule. The vector also contains appropriate promoter(s)/enhancer(s). Also described herein is a method to construct a vector into which these genetic elements have been incorporated.

Regarding the reverse transcriptase gene which is the first component of the cassette, the reverse transcriptase gene from the Moloney Murine Leukemia Virus (MoMuLV) was used to advantage in the examples described. Many other retroviral reverse transcriptase genes may be used to advantage in the cassette of the present invention, it being preferred that the reverse transcriptase gene is regulated by an appropriate upstream promoter/enhancer such as the Cytomegalovirus (CMV) or Rouse Sarcoma Virus (RSV) promoter for expression in eukaryotic cells.

The reverse transcriptase gene also preferably includes a downstream polyadenylation signal sequence so that the mRNA produced from the reverse transcriptase gene includes a 3' poly(A) tail for mRNA stability. As known to those skilled in the art, multiple poly(A) tails are available and are routinely used for production of

expressed eukaryotic genes. The reverse transcriptase produced in the cell synthesizes a complementary DNA (cDNA) from the primary mRNA transcript transcribed from the template encoding the genetic element that includes the sequence of interest as described below. The RNase H activity of the reverse transcriptase, along with endogenous RNase H activity within the cell, degrades the mRNA component of the heteroduplex RNA/cDNA hybrid to produce ssDNA *in vivo*.

The second component included in the cassette encodes a nucleic acid sequence that provides the template for synthesis of ssDNA in target cells. It is this element that includes the sequence of interest. As is the case for the above reverse transcriptase gene, this genetic element is preferably regulated by an appropriate wide spectrum or tissue-specific promoter(s)/enhancer(s), such as the SV-40 promoter, or combination of promoter(s)/enhancer(s), located upstream of the genetic element. Those skilled in the art who have the benefit of this disclosure will also recognize that a number of tissue-specific or wide spectrum promoters/enhancers, or combinations of promoters/enhancers may be used to advantage to regulate the reverse transcriptase gene and sequence of interest. Although a list of all available promoters/enhancers is not needed to exemplify the invention, the promoters/enhancers may be constitutive or inducible and may include the CMV or RSV (non-cell type specific) or GFAP (tissue specific) promoters/enhancers listed here and many other viral or mammalian promoters. Representative promoters/enhancers that are appropriate for use in connection with the present invention may include, but are not limited to, HSVtk (McKnight *et al.*, 217 Science 316, 1982), human beta-globulin (Breathnach *et al.*, 50 Ann. Rev. of Biochem. 349, 1981), beta-actin (Kawamoto *et al.*, 8 Mol. Cell Biol. 267, 1988), rat growth hormone (Larsen *et al.*, 83 Proc. Natl. Acad. Sci. U.S.A. 8283, 1986), MMTV (Huang *et al.*, 27 Cell 245 1981), adenovirus 5 E2 (Imperiale, *et al.*, 4 Mol. Cell. Biol. 875, 1984), SV40 (Angel *et al.*, 49 Cell 729, 1987), a-2-macroglobulin (Kunz, *et al.*, 17 Nucl. Acids Res. 1121, 1989), MHC class I gene H-2kb (Blanar *et al.*, 8 EMBO J. 1139, 1989), and thyroid stimulating hormone (Chatterjee *et al.*, 86 Proc. Natl. Acad. Sci. U.S.A. 9114, 1989).

For expression in eukaryotic cells, the sequence of interest is followed downstream by a genetic element encoding for a primer-binding site (PBS) for initiation of cDNA synthesis by reverse transcription. The PBS is a sequence that is complementary to a

transfer RNA (tRNA) which resides within the eukaryotic target cell. The PBS included in the presently preferred set of genetic elements described herein was taken from the actual 18 nucleotide sequence region of MoMuLV. However, any PBS that is matched to the reverse transcriptase that comprises the set of genetic elements may be utilized for this
5 purpose. Multiple copies of the sequences of interest, each with its corresponding PBS, can be incorporated into the vector for delivery to a cell in accordance with the method of the present invention if desired, for example, for use in delivering anti-sense sequences to various regions of a gene within the target cell.

The mRNA primary transcript transcribed from the genetic element acts as the
10 template used by the reverse transcriptase described above to synthesize and process the sequence of interest, which as noted above, can be any desired ssDNA. The mRNA primary transcript contains a primer binding site (PBS) downstream to the sequence of interest. The PBS is exclusively recognized by a "primer tRNA." To those skilled in the art, tRNAs are endogenous to cells. Each tRNA has the ability to recognize a unique
15 sequence (i.e., codon) on the mRNA transcript coding for an amino acid, and has the ability to covalently link to a specific amino acid (i.e., the tRNA becomes "charged" when bound to a specific amino acid). However, a "primer tRNA" when bound to the mRNA transcript PBS and not covalently linked (i.e., "uncharged") with an amino acid, may be used to initiate ssDNA synthesis by the reverse transcriptase. For example, the MoMuLV
20 reverse transcriptase used in the examples described herein, recognizes and uses an uncharged lysine tRNA that in turn recognizes and binds to its unique sequence in the PBS. Thus, each PBS incorporated into the vector must contain the unique sequence recognized by the primer tRNA, and the primer tRNA must be one that is recognized by the particular reverse transcriptase utilized.

25 It is preferred that the vector contain other specialized genetic elements to facilitate the identification of cells that carry the set of genetic elements of the present invention and/or to increase the level of expression of the sequence of interest. The specialized genetic elements include selectable marker genes so that the vector can be transformed and amplified in a prokaryotic system. For example, the most commonly used
30 selectable markers are genes that confer to the bacteria (e.g., *E. coli*) resistance to antibiotics such as ampicillin, chloramphenicol, kanamycin (neomycin), or tetracycline. It

is also preferred that the vector contain specialized genetic elements for subsequent transfection, identification and expression in a eukaryotic system. For expression in eukaryotic cells, multiple selection strategies (e.g., Chinese Hamster Ovarian: CHO) may be used that confer to the cell resistance to an antibiotic or other drug or alter the 5 phenotype of the cell such as morphological changes, loss of contact inhibition, or increased growth rate. Selectable markers used in eukaryotic systems include, but are not limited to, resistance markers for Zeocin, resistance to G418, resistance to aminoglycoside antibiotics, or phenotypic selection markers such β -gal or green fluorescence protein.

It will also be evident to those skilled in the art from this description that the linear 10 ssDNA can be formed into an intact stem-loop ssDNA structure by the addition of inverted tandem repeats flanking the sequence of interest that form the "stem" portion after duplex formation. The stem-loop structure can function similarly in many applications as the linear ssDNA form. Such a ssDNA structure may be more resistant to intracellular nucleases by retaining the "ends" of a ssDNA in double stranded form.

15 It will also be evident to those skilled in the art that the stem (duplex DNA) can be designed to contain a predetermined sequence or sequences (i.e., "aptamers") that are recognized and bound by specific DNA-binding proteins. Among other uses, such a stem structure is used in the cell as a competitor to titer out a selected protein(s) that regulate specific gene expression. For example, a ssDNA stem-loop of the present invention in a 20 cell such that the "stem" contains a binding site for a selected transcription factor such as adenovirus E1a. Adenovirus E1a, like other oncogenes, modulates gene expression of several adenoviral and cellular genes by affecting the activity of cell-encoded transcription factors resulting in changing normal cells to transformed cells. (Jones *et al.*, Genes Dev. 2, 267-281 (1988)). The duplex structure of the stem thus functions to "bind up" the 25 factor, preventing the protein from binding a promoter and thus inhibiting the expression of a particular deleterious gene. To those skilled in the art, it will be clear that the duplex stem structure may optionally contain multiple binding sites, for example, sites which are recognized by various transcription factors that actively regulate expression of particular gene. For example, adenovirus E1a has been found to repress transcription of the 30 collagenase gene via the phorbol ester-responsive element, a promoter element responsible for the induction of transcription by 12-O-tetradecanolyphorbol 13-acetate (TPA), by a

number of other mitogens, and by the *ras*, *mos*, *src*, and *trk* oncogenes. The mechanism involves inhibition of the function of the transcription factor family AP-1. Offringa *et al.*, 62 Cell 527-538 (1990).

In another aspect which will be recognized by those skilled in the art, the present invention is used to construct complex secondary ssDNA structures in the loop portion of the DNA transcript produced in accordance with the present invention. Such secondary structure is engineered to serve any of several functions. For instance, the sequence of interest optionally includes (but is not limited to) a sequence which is incorporated into the loop portion of the single-stranded cDNA transcript to form so-called "clover leaf" or "crucible" like structures such as those found in the long terminal repeats of adenoassociated virus or in retrotransposons. Under correct circumstances, such structure is integrated in site-specific manner into the host genome.

Because a vector incorporating the set of genetic elements of the present invention is adaptable for incorporation into multiple commercially available delivery vectors for mammalian and human therapeutic purposes, multiple delivery routes are feasible depending upon the vector chosen for a particular target cell. For example, viral vectors are presently the most frequently used means for transforming the patient's cells and introducing DNA into the genome. In an indirect method, viral vectors, carrying new genetic information, are used to infect target cells removed from the body, and these cells are then re-implanted (i.e., *ex vivo*). Direct *in vivo* gene transfer into postnatal animals has been reported for formulations of DNA encapsulated in liposomes and DNA entrapped in proteoliposomes containing viral envelope receptor proteins (Nicolau *et al.*, Proc. Natl. Acad. Sci USA 80:1068-1072 (1983); Kaneda *et al.*, Science 243:375-378 (1989); Mannino *et al.*, Biotechniques 6:682-690 (1988). Positive results have also been described with calcium phosphate co-precipitated DNA (Benvenisty and Reshef, Proc. Natl. Acad. Sci USA 83:9551-9555 (1986)). Such systems include intravenous, intramuscular, and subcutaneous injection, as well as direct intra-tumoral and intra-cavitory injections. The set of genetic elements, when incorporated into the vector of choice, can also be administered through transmucosal, rectal, oral, or inhalation-type methods of delivery.

The vector incorporating the set of genetic elements of the present invention is advantageously employed to deliver antisense, sense, triplex, or any other single-stranded

nucleotide sequence of interest, using known digestion and ligation techniques to splice the particular sequence of interest into the vector in the presence or absence of inverted tandem repeats. Those skilled in the art who have the benefit of this disclosure will also recognize that the above-described signals used for expression within eukaryotic cells may 5 be modified in ways known in the art depending upon the particular sequence of interest. The most likely change is to change the promoter so as to confer advantageous expression characteristics on the sequence of interest in the system in which it is desired to express the sequence of interest. There are so many possible promoters and other signals, and they are so dependent on the particular target cell for which the sequence of interest has 10 been selected, that it is impossible to list all the potential enhancers, inducible and constitutive promoter systems, and/or poly(A) tailing systems which may be preferred for a particular target cell and sequence of interest.

The present invention is also utilized to produce inhibitory nucleic acids for use in therapeutics *in vivo* or *in vitro*. Inhibitory nucleic acids may be ssDNA synthesized from the 15 mRNA template or the mRNA template itself, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA--RNA, a DNA-DNA, or RNA-DNA duplex or triplex is formed. More commonly, these nucleic acids are often termed "antisense" because they are usually complementary to the sense or coding strand of the gene, but the "sense" sequence is also utilized in the cell for therapeutic purposes. For 20 example, the identification of oligonucleotides that specifically bind to biomolecules that do not normally bind to RNA or DNA has now been demonstrated for a number of biomolecules that vary widely in size, structure and composition. These molecules include: (1) thrombin, a multifunctional regulatory protein that converts fibrinogen to fibrin in the process of clot formation; (2) bradykinin, a nonapeptide kinin involved in blood pressure regulation and 25 implicated in hypotension; (3) PGF2.alpha., a prostaglandin or fatty acid derivative that exhibits hormonal activity. Additionally, the interaction of oligonucleotides with biomolecules whose natural biological function is primarily extracellular has now been demonstrated. U.S. Pat. No. 5,840,867. The term "inhibitory nucleic acids" as used herein, therefore, refers to both "sense" and "antisense" nucleic acids.

30 By binding to the target nucleic acid, an inhibitory nucleic acid inhibits the function of the target nucleic acid. This inhibitory effect results from, for example, blocking DNA

transcription, processing or poly(A) addition to mRNA, DNA replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradation. Inhibitory nucleic acid methods therefore encompass a number of different approaches to altering expression of genes. An example of an antiherpes virus inhibitory nucleic acid is ISIS 2922
5 (ISIS Pharmaceuticals, Carlsbad, CA) which has activity against CMV (see Biotechnology News 14:5). These different types of inhibitory nucleic acid technologies are described in Helene, C. and Toulme, J. (1990) Biochim. Biophys. Acta. 1049:99-125, which is referred to hereinafter as "Helene and Toulme."

In brief, inhibitory nucleic acid therapy approaches can be classified into (1) those that target DNA sequences, (2) those that target RNA sequences (including pre-mRNA and mRNA), (3) those that target proteins (sense strand approaches), and (4) those that cause cleavage or chemical modification of the target nucleic acids. The first approach contemplates several categories. Nucleic acids are designed to bind to the major groove of the duplex DNA to form a triple helical or "triplex" structure. Alternatively, inhibitory nucleic acids are designed
10 to bind to regions of single stranded DNA resulting from the opening of the duplex DNA during replication or transcription. More commonly, inhibitory nucleic acids are designed to bind to mRNA or mRNA precursors. Inhibitory nucleic acids are used to prevent maturation of pre-mRNA. Inhibitory nucleic acids may be designed to interfere with RNA processing, splicing or translation. In the second approach, the inhibitory nucleic acids are targeted to
15 mRNA. In this approach, the inhibitory nucleic acids are designed to specifically block translation of the encoded protein. Using this second approach, the inhibitory nucleic acid can be used to selectively suppress certain cellular functions by inhibition of translation of mRNA encoding critical proteins. For example, an inhibitory nucleic acid complementary to regions of c-myc mRNA inhibits c-myc protein expression in a human promyelocytic leukemia cell line,
20 HL60, which overexpresses the c-myc proto-oncogene. See Wickstrom E. L., *et al.* (1988) PNAS 85:1028-1032 and Harel-Bellan, A., *et al.* (1988) Exp. Med. 168:2309-2318. As described in Helene and Toulme, inhibitory nucleic acids targeting mRNA have been shown to work by several different mechanisms to inhibit translation of the encoded protein(s).

The inhibitory nucleic acids introduced into the cell can also utilize the third
30 approach of designing the "sense" strand of the gene or mRNA to trap or compete for the enzymes or binding proteins involved in mRNA translation, as described in Helene and

Toulme. Lastly, the inhibitory nucleic acids is used to induce chemical inactivation or cleavage of the target genes or mRNA. Chemical inactivation occurs by the induction of crosslinks between the inhibitory nucleic acid and the target nucleic acid within the cell.

In another embodiment, the present invention takes the form of a kit comprised of 5 a plasmid having the above-described reverse transcriptase gene cloned therein as well as a multiple cloning site (MCS) into which the user of the kit inserts a particular sequence of interest, which may or may not include the above-described inverted tandem repeats in accordance with the user's intended result. The MCS is upstream from the genetic element encoding the primer binding site. The resulting plasmid is then purified from the 10 cell culture in which it is maintained, lyophilized or otherwise preserved for packaging and shipping to the user. The kit preferably also includes the restriction endonuclease(s) for the MCS into which the sequence of interest is to be cloned, the ligases and other enzymes for inserting the sequence of interest into the plasmid, and a map of the plasmid, along with suitable reaction buffers.

15 Except where otherwise indicated, standard techniques are described by Seabrook, *et al.* (1989) (J. Seabrook, *et al.*, Molecular Cloning: A Laboratory Manual (2nd Ed.), Cold Spring Harbor Press (1989), hereinafter referred to as "Maniatis, *et al.* (1989)") were utilized in the examples set out below. Several experimental designs are presented to illustrate the method of producing ssDNA *in vivo*.

20 EXAMPLES

The following examples are provided for illustrative purposes only and are not intended to limit the scope of the invention.

Materials

25 The plasmid pcDNA3.1/Zeo⁺ was purchased from Invitrogen Corp. (San Diego, CA) and plasmid PBK-RSV from Statagene (La Jolla, CA). Oligodeoxyribonucleotides (ODN) were synthesized by Midland Certified Reagent Co. (Midland, TX). Polymerase chain reactions (PCR) were carried out using Taq DNA polymerase purchased from Boehringer Mannheim Corp. (Indianapolis, IN) in a Robo-gradient thermal cycler (Stratagene (La Jolla, CA)). Restriction endonucleases and T4 DNA ligase were obtained from Boehringer 30 Mannheim Corp. (Indianapolis, IN). The ODNs used are listed in the attached Sequence Listing.

Example 1

In vivo Synthesis of ssDNA in Eukaryotic Cells

The following *in vivo* experiments were designed to determine whether ssDNA could be produced in intact cells. To control expression of the genetic elements cloned into the 5 plasmid in these host cells, the plasmid utilized included the RSV promoter. However, those skilled in the art who have the benefit of this disclosure will recognize that any of the eukaryotic promoters listed above can be used for this purpose.

Plasmid Constructs. The cloning vector pssXB and the plasmids containing the sequences to be expressed as single-stranded DNA were constructed from a common 10 intermediate construct. The host strain for these manipulations was XL1-Blue MRF' (Stratagene, La Jolla, CA).

In the first cloning stage, to obtain the common intermediate, the vector pcDNA3.1Zeo^r (Invitrogen, San Diego, CA) was digested with the restriction enzymes *Nhe* I and *Apa* I. The double-stranded oligodeoxyribonucleotide having compatible *Nhe* I and *Apa* I 15 ends, which is formed by annealing the synthetic, single-stranded oligodeoxynucleotides ODN-PMMV(+) and ODN-PMMV(-) (see Table I), was ligated into the digested pcDNA3.1Zeo^r to give pcPMMV. This insert contains the Moloney Murine leukemia virus (MoMuLV) reverse transcriptase promoter region. It also contains two *Not* I sites, unique in pcPMMV. In 20 this construct and in the plasmids deriving from this construct, the strands designated (+) are positioned to be transcribed into RNA from the cytomegalovirus (CMV) promoter of pcDNA3.1/Zeo(+).

The plasmid pssDNA-Express-A (pssXA), containing genes for MoMuLV reverse transcriptase, was constructed from the vector pBK-RSV (Stratagene, La Jolla, CA), also using XL-1 Blue MRF' as the host strain. A mouse cell line expressing MoMuLV was 25 obtained from the American Type Culture Collection (ATCC #CRL-1858). Virus RNA was isolated and reverse transcribed from ODN-RT (-) (Table I). The reverse transcript was then PCR amplified according to the manufacturer's instructions using a kit from Promega (Madison, WI), primers ODN-RT (+) and ODN-RT (-), and digested with *Sac* I and *Hind* III (sites for these restriction endonucleases are present in the 5' and 3' primers, respectively). The 2.4 kb 30 product obtained includes the sequence of the MoMuLV genome between positions 2546 and

4908. The mature virus reverse transcriptase peptide is encoded by the sequence between positions 2337 and 4349 (Petropoulos, C.J. Retroviral taxonomy, protein structure, sequences and genetic maps. In: *Retroviruses*, 757, Appendix 2, Coffin, J.M. (Ed.). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA 1997), but peptides truncated at the 5 amino terminus retain full activity (Sun, *et al.* (1998)).

The pBRK-RSV vector was digested with *Xba* I and *Nhe* I, which removes the *lac* promoter region. The *Nhe* I end was converted to a *Sac* I end using the linker formed by annealed oligodeoxynucleotides ODN-N>S (+) and ODN-N>S (-). The reverse transcriptase amplimers were ligated through the *Hind* III sites and this construct was subsequently ligated 10 between the *Sac* I and *Xba* I sites of pBK-RSV to give pBK-RSV-RT.

Those skilled in the art will recognize that the set of genetic elements comprising the present invention are also expressed from a single plasmid made by a fusion of, for instance, the pc3.1DNA/Zeo-derived plasmids and the pBK-RSV-derived plasmids such that fused plasmids encode the ss-cDNA-encoding genetic element, the Mo-MuLV-RT gene, and the PBS. pBK-15 RSV-RT/MboL is digested with *Nsi*I to release a 5.3kb fragment containing the Mo-MuLV-RT gene with an intervening his-pro linker and associated regulatory elements. The 5.3kb DNA fragment is ligated to a linker containing an internal EcoRI site and digested with EcoRI. The pc3.1/Zeo/N-M and the derivative plasmids containing test sequences are digested with BglII, which recognizes a unique site on pc3.1DNA/Zeo in the cytomegalovirus 20 enhancer/promoter (P CMV). The BglII ends are ligated to Seq. ID 15 and Seq. ID 16, which contain an internal EcoRI site. After digestion with EcoRI, the 5.3kb fragment is ligated to pc3.1/Zeo/N-M and derivatives to generate the plasmid.

Tissue culture studies. Stable and transient transfections are carried out by using lipofectant (Boehringer Mannheim Corp.) using the manufacturer's accompanying instructions. 25 All plasmid constructs were transfected into Cos-7, U251 and HeLa cell lines. Assays for ssDNA were performed by PCR and by dot-blot analyses 24-48 hours after transfection. Reverse transcriptase activity was assayed using the RT-PCR assay developed by Silver, *et al.* (Silver, J., *et al.* 21 Nucleic Acids Res. 3593-4 (1993)). The ss-cDNA is isolated from cells transfected 48-72-hr earlier using triazol reagent (Gibco Life Technologies, Gaithersburg, 30 MD). Assays for specific ss-cDNA species are carried out by both PCR based assays for

internal fragment and by denatured single stranded gel electrophoresis with subsequent nylon blotting and probing with an internal biotin-labeled probe.

The experiments described above demonstrate a method of production of ssDNA *in vivo* by multiple stepwise reactions using eukaryotic reverse transcriptase reactions and various 5 cDNA priming reactions. Any nucleotide sequence of interest is produced by this method in a prokaryotic or eukaryotic cell. The cells were actually co-transfected with two plasmids, one plasmid carrying the genetic elements encoding the sequence of interest and primer binding site for reverse transcriptase, shown in Fig. 1A and the other carrying the gene for reverse transcriptase shown in Fig. 1B. Those skilled in the art, however, will recognize that a single 10 plasmid including the genetic elements encoding the sequence of interest and PBS for reverse transcriptase, and the gene for reverse transcriptase also can be used for this purpose (Fig. 1C).

Example 2

Reverse Transcriptase Activity in Transformed Cells

To determine the presence of reverse transcriptase activity in extracts of cells 15 containing the pBK-RSV-RT construct, the following assay is used. This assay relies upon reverse transcriptase activity in protein extracts of transformed cells to produce a DNA copy of the Brome Mosaic Virus RNA genome (Silver, *et al.*, 1993). The replication cycle of this virus does not involve a DNA intermediate, eliminating the possibility that an amplification product could be produced without prior reverse transcription.

20

Example 3

Demonstration of the presence of single-stranded DNA in Transformed Mammalian Cells.

A PCR strategy is used to detect single-stranded DNA in transformed cells. The product obtained from RNA extraction procedures, which presumably includes the single- 25 stranded DNA is used as a template in PCR amplifications using primers specific for the expected single-stranded DNA molecule, which is not otherwise present in the cells. A band of the expected size is produced from untreated RNA/ssDNA preparations and from such preparations that were treated with RNAase A. Use of preparations treated with S1 nuclease, a highly specific, single-stranded DNA endonuclease, does not result in an amplified product.

Example 4

A method and pharmaceutical preparation
for diagnosing and treating pathological conditions
related to a dopamine receptor abnormality.

5 Abnormal activity of the dopaminergic nervous system has been implicated in a number of motor and behavioral disorders including Parkinson's disease, Huntington's disease, tardive dyskinesia, certain forms of schizophrenia and other dystonias and dyskinésias. Dysfunctions of the dopaminergic system may be caused either by a reduced or increased activity of the dopaminergic system or by the inability of the systems to be modulated by a
10 changing external or internal environment.

For a patient suffering from one of the above mentioned disorders, a plasmid is constructed to include a sequence of interest that generates an antisense oligonucleotide capable of binding specifically to an expression-controlling sequence of a nucleic acid encoding the dopamine receptor. The plasmid is administered under conditions whereby the plasmid
15 enters cells expressing the dopamine receptor and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, thereby selectively controlling expression of one or more dopamine receptor subtypes, and alleviating the pathological conditions related to their expression. Efficacy is tested in accordance with the method described in U.S. Patent No. 5,840,708.

20

Example 5

Inhibitory nucleotides to Kaposi's sarcoma-associated herpesvirus (KSHV)
virion protein 26 (VP26)

Kaposi's sarcoma-associated herpes virus (KSHV) is a new human herpes virus (HHV8) believed to cause Kaposi's sarcoma (KS). Kaposi's sarcoma is the most common
25 neoplasm occurring in persons with acquired immunodeficiency syndrome (AIDS). Approximately 15-20% of AIDS patients develop this neoplasm which rarely occurs in immunocompetent individuals. Epidemiologic evidence suggests that AIDS-associated KS (AIDS-KS) has an infectious etiology. Gay and bisexual AIDS patients are approximately twenty times more likely than hemophiliac AIDS patients to develop KS, and KS may be
30 associated with specific sexual practices among gay men with AIDS. KS is uncommon among adult AIDS patients infected through heterosexual or parenteral HIV transmission, or among

pediatric AIDS patients infected through vertical HIV transmission. Agents previously suspected of causing KS include cytomegalovirus, hepatitis B virus, human papillomavirus, Epstein-Barr virus (EBV), human herpesvirus 6, human immunodeficiency virus (HIV), and Mycoplasma penetrans. Non-infectious environmental agents, such as nitrite inhalants, also 5 have been proposed to play a role in KS tumorigenesis. Extensive investigations, however, have not demonstrated an etiologic association between any of these agents and AIDS-KS.

10 Virion protein 26 (VP26) is a component of the nucleocapsid structure in most herpes viruses. This structure serves as a delivery mechanism for the viral genome as it is spread from one infected cell to another. As part of the original infecting virus, it is recognized 10 as a major antigen by the immune system and can therefore be used to screen for antibodies to the herpes virus in patient sera and as a vaccine.

15 For an infected patient, a plasmid is constructed using the methods described above to include a sequence of interest. The sequence of interest is an isolated nucleic acid molecule which encodes KSHV virion protein 26 or antisense or triplex oligonucleotide molecule as 15 described in U.S. Patent No. 5,840,708. The plasmid is administered under conditions whereby the plasmid enters infected cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, thereby selectively controlling expression of KSHV virion 20 protein 26, and alleviating the pathological conditions related to expression.

20

Example 6

Inhibitory nucleotides to modulate the expression of IL-8 and/or IL-8 Receptor to control growth, metastasis and/or angiogenesis in tumors.

Interleukin-8 (IL-8, neutrophil activating protein-1, or NAP-1) is a member of C-X-C 25 chemokine family of related cytokines having broad involvement in inflammatory responses, tissue injury, growth regulation and cellular adhesion. Cerretti, D. P., *et al.*, Molecular Characterization of Receptors for Human Interleukin-8, GRO/Melanoma Growth-Stimulatory Activity and Neutrophil Activating Peptide-2, *Molecular Immunology*, 30(4), 359-367 (1993); and Koch, A. E., *et al.*, In situ expression of cytokines and cellular adhesion molecules in the skin of patients with systemic sclerosis, *Pathobiology*, 61(5-6), 239-46 (1993). IL-8 has also 30 been shown to have a potent stimulatory effect on angiogenesis. See, e.g., Koch, A. E.,

Interleukin-8 as a Macrophage-Derived Mediator of Angiogenesis, Science, 258, 1798-1800 (1992).

It is known that IL-8 is produced by a variety of normal human somatic cells including monocytes/macrophages, dermal fibroblasts, vascular endothelial cells, keratinocytes, 5 and mesangeal cells. Yasumoto, K., et al., Tumor Necrosis Factor Alpha and Interferon Gamma Synergistically Induce Interleukin 8 Production in a Human Gastric Cancer Cell Line Though Acting Concurrently on AP-1 and NF-kB-like Binding Sites of the Interleukin 8 Gene, J. of Biological Chemistry, 267(31), 22506-11 (1992). Apparently, such cells produce IL-8 only when stressed, and not under conditions of normal growth and homeostasis. Factors that 10 induce IL-8 production include inflammation, IL-1, TNF, LPS and thrombin. It is also known that IL-8 is commonly secreted by tumor cells. Because of its effects on growth, it is suspected that IL-8 has a significant role in the metastatic spread of melanoma and other cancers.

IL-8 is a ligand for cell-membrane IL-8 Receptor, and it is thought that interaction between IL-8 and IL-8 Receptor is required for IL-8 action. Two IL-8 receptor genes have 15 been identified so far, IL-8 Receptor type A and type B. Both genes belong to the so-called seven transmembrane domain, G protein-coupled receptor family. Receptor A has been shown to be activated by IL-8, and receptor B has been shown to be activated by IL-8 as well as other cytokines belonging to C-X-C family including Melanoma Growth Stimulatory Activity (MGSA).

20 The role and function of IL-8 Receptor B present in cancer and other tumor cells is not fully elucidated. There is, however, evidence that activation of IL-8R B (1) is involved in the mechanism of growth regulation of melanoma and tumorigenic fibroblasts; (2) is associated with transformation of lung cells by asbestos, and (3) correlates with metastatic potential of melanoma.

25 Given the growth stimulatory effect of IL-8 on cells responsive to various tumor growth factors, it would be advantageous to provide antisense oligonucleotides which modulate expression of either IL8 or IL-8 Receptor in cancers in vivo. It would be particularly advantageous to provide oligonucleotides which are effective against lung cancer and melanoma because each of these cancers produce their own growth factors.

30 There are at least two major types of lung cancer, small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). SCLC comprises approximately one-fourth of

the cases, expresses neuroendocrine markers, and generally metastasizes early to lymph nodes, brain, bones, lung and liver. NSCLC comprises the majority of the remaining lung tumor types, and includes adeno-carcinoma, squamous cell carcinoma, and large cell carcinoma. NSCLC is characterized by epithelial-like growth factors and receptors, and is locally invasive.

5 Melanoma cells, unlike normal melanocytes, can proliferate in the absence of exogenous growth factors. This independence apparently reflects the production of growth factor and cytokines for autocrine growth stimulation, including TGF- α , TGF-, platelet-derived growth factor A and B chains, basic fibroblast growth factor, IL-8, IL-6, IL-1, granulocyte macrophage colony stimulating factor, and MGSA. Guo Y, *et al.*, Inhibition of
10 Human Melanoma Growth and Metastasis in Vivo by Anti-CD44 Monoclonal Antibody. Cancer Res., 54, 1561-1565 (1994).

For a patient suffering from any of the above diseases, a plasmid is constructed using the methods described above to include a sequence of interest. The sequence of interest is an isolated nucleic acid molecule as described in U.S. Patent No. 5,849,903. To control growth, 15 metastasis and/or angiogenesis, the plasmid is administered (e.g., inhalation or direct injection into solid tumors) under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, thereby selectively controlling expression of IL-8 receptors, and alleviating the pathological conditions related to expression.

20 Example 7

Antisense oligonucleotide inhibition of cytomegalovirus infection.

Cytomegaloviruses (CMVs) are ubiquitous in nature and are the most common causes of intrauterine infection. Congenital infection is common in newborns of infected mothers. In some populations, as much as 10% of children display perinatal infections. In a 25 small percentage of newborns, the infection is virulent, involving multiple organs. Pronounced involvement of the reticuloendothelial and central nervous system is typical; and the infection is a major cause of mental retardation. Careful testing demonstrates that as many as 50% of severely, prenatally infected adults may display neuropsychiatric disease or deafness. Although extraneuronal organs are usually spared chronic morbidity, the virus can be detected in the kidney 30 for years.

A plasmid is constructed using the methods described above to include a sequence of interest encoding for an inhibitory nucleotide. Oligonucleotides having a sequence of nucleotide bases specifically hybridizable with a selected sequence of a cytomegalovirus DNA or RNA are described in U.S. Patent No. 5,442,049. The plasmid is administered to the patient under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, thereby selectively controlling replication of CMV, and alleviating the pathological conditions related to CMV infection. This plasmid is used either prophylactically or therapeutically to reduce the severity of disease caused by CMV.

10

Example 8

Oligonucleotides specifically hybridizable with RNA or DNA
deriving from a gene corresponding to one of the open reading frames
UL5, UL8, UL9, UL20, UL27, UL29, UL30, UL42, UL52 and IE175
of herpes simplex virus type 1.

15 Oligonucleotides are designed to be specifically hybridizable with DNA or even more preferably, RNA from one of the species herpes simplex virus type 1 (HSV-1), herpes simplex virus type (HSV-2), cytomegalovirus, human herpes virus 6, Epstein Barr virus (EBV) or varicella zoster virus (VZV). Such oligonucleotides are conveniently and desirably presented as a pharmaceutical composition in a pharmaceutically acceptable carrier as described in U.S. 20 Patent No. 5,514,577.

For a patient suffering from any of the above infections, a plasmid is constructed using the methods described above to include a sequence of interest. The sequence of interest is an isolated nucleic acid molecule as described in U.S. Patent No. 5,514,577. The plasmid is administered (e.g., inhalation or direct injection into solid tumors) under conditions whereby 25 the plasmid enters cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, from one of the species herpes simplex virus type 1 (HSV-1), herpes simplex virus type (HSV-2), cytomegalovirus, human herpes virus 6, Epstein Barr virus (EBV) or varicella zoster virus (VZV) thereby selectively controlling virus infection, and alleviating the 30 pathological conditions related to infection.

Example 9

Antisense oligonucleotides to proto-oncogenes, and in particular to the c-myb gene, and the use of such oligonucleotides as antineoplastic and immunosuppressive agents.

The proto-oncogene c-myb is the normal cellular homologue of the avian myeloblastosis virus-transforming gene v-myb. The c-myb gene codes for a nuclear protein expressed primarily in hematopoietic cells. It is a proto-oncogene, that is, it codes for a protein which is required for the survival of normal, non-tumor cells. When the gene is altered in the appropriate manner, it has the potential to become an oncogene. Oncogenes are genes whose expression within a cell provides some function in the transformation from normal to tumor cell. An example is the human c-myb gene which has been isolated, cloned, and sequenced. Majello et al, Proc. Natl. Acad. Sci. U.S.A. 83, 9636-9640 (1986).

A plasmid is constructed using the methods described above to include a sequence of interest encoding for an inhibitory nucleotide. Oligonucleotides having a sequence of nucleotide bases specifically hybridizable with a selected sequence of the DNA or RNA as are described in U.S. Patent No. 5,098,890. The plasmid is administered to the patient under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide thus acting as an antineoplastic or immunosuppressive agent.

Example 10

Antisense oligonucleotides Against ICAM-1 Gene Expression in Interleukin-1 beta-Stimulated Cells.

It is has been hoped that inhibitors of ICAM-1, VCAM-1 and ELAM-1 expression would provide a novel therapeutic class of anti-inflammatory agents with activity towards a variety of inflammatory diseases or diseases with an inflammatory component such as asthma, rheumatoid arthritis, allograft rejections, inflammatory bowel disease, various dermatological conditions, and psoriasis. In addition, inhibitors of ICAM-1, VCAM-1, and ELAM-1 may also be effective in the treatment of colds due to rhinovirus infection, AIDS, Kaposi's sarcoma and some cancers and their metastasis. To date, there are no known therapeutic agents which effectively prevent the expression of the cellular adhesion molecules ELAM-1, VCAM-1 and ICAM-1. The use of neutralizing monoclonal antibodies against ICAM-1 in animal models provide evidence that such inhibitors if identified would have therapeutic benefit for asthma; Wegner et al., Science 1990, 247, 456-459, renal allografts; Cosimi et al., J. Immunol. 1990,

144, 4604-4612, and cardiac allografts; Isobe *et al.*, Science 1992, 255, 1125-1127. The use of a soluble form of ICAM-1 molecule was also effective in preventing rhinovirus infection of cells in culture. Marlin *et al.*, 344 Nature 70-72 (1990).

Current agents which affect intercellular adhesion molecules include synthetic peptides, monoclonal antibodies, and soluble forms of the adhesion molecules. To date, synthetic peptides which block the interactions with VCAM-1 or ELAM-1 have not been identified. Monoclonal antibodies may prove to be useful for the treatment of acute inflammatory response due to expression of ICAM-1, VCAM-1 and ELAM-1. However, with chronic treatment, the host animal develops antibodies against the monoclonal antibodies thereby limiting their usefulness. In addition, monoclonal antibodies are large proteins which may have difficulty in gaining access to the inflammatory site. Soluble forms of the cell adhesion molecules suffer from many of the same limitations as monoclonal antibodies in addition to the expense of their production and their low binding affinity. Thus, there is a long felt need for molecules which effectively inhibit intercellular adhesion molecules. Antisense oligonucleotides avoid many of the pitfalls of current agents used to block the effects of ICAM-1, VCAM-1 and ELAM-1.

PCT/US90/02357 (Hession, *et al.*) discloses DNA sequences encoding Endothelial Adhesion Molecules (ELAMs), including ELAM-1 and VCAM-1 and VCAM-1b. A number of uses for these DNA sequences are provided, including (1) production of monoclonal antibody preparations that are reactive for these molecules which may be used as therapeutic agents to inhibit leukocyte binding to endothelial cells; (2) production of ELAM peptides to bind to the ELAM ligand on leukocytes which, in turn, may bind to ELAM on endothelial cells, inhibiting leukocyte binding to endothelial cells; (3) use of molecules binding to ELAMS (such as anti-ELAM antibodies, or markers such as the ligand or fragments of it) to detect inflammation; (4) use of ELAM and ELAM ligand DNA sequences to produce nucleic acid molecules that intervene in ELAM or ELAM ligand expression at the translational level using antisense nucleic acid and ribozymes to block translation of a specific mRNA either by masking mRNA with antisense nucleic acid or cleaving it with a ribozyme.

A plasmid is constructed using the methods described above to include a sequence of interest encoding for an inhibitory nucleotide for ICAM-1, VCAM-1 or ELAM-1. Oligonucleotides having a sequence of nucleotide bases specifically hybridizable with a selected

sequence of ICAM-1, VCAM-1 or ELAM-1 DNA or RNA are described in U.S. Patent No. 5,843,738. The plasmid is administered to the patient under conditions whereby the plasmid enters cells and generates the inhibitory nucleotide. The inhibitory nucleotide binds specifically to expression-controlling sequences of such RNA molecules, or encoding sequences, thereby selectively controlling the expression of ICAM-1, VCAM-1 or ELAM-1, and alleviating the pathological conditions related to ICAM-1, VCAM-1 and ELAM-1 expression. This plasmid is used either prophylactically or therapeutically to reduce the severity of inflammation caused by ICAM-1, VCAM-1 and ELAM-1.

Example 11

10 Protein-Binding Oligonucleotides (Aptamers) Specifically Bind Target Molecules

The field of rational drug design using biomolecule targeting and aptamer development utilizes oligonucleotides to bind to specific proteins and thus interfere with their function. Described in U.S. Pat. No. 5,840,867, are aptamers to biomolecular targets such as proteins in general, and thrombin in particular. The novel compounds and methods disclosed may be applied broadly to biotechnology diagnostics and therapeutics.

Conventional methods of detection and isolation of proteins and other molecules have employed antibodies and the like which specifically bind such substances. Recently, however, the de novo design of specifically binding oligonucleotides for non-oligonucleotide targets that generally bind nucleic acids has been described. See, e.g., Blackwell, T. K., et al., Science 20 (1990) 250:1104-1110; Blackwell, T. K., et al., Science (1990) 250:1149-1152; Tuerk, C., and Gold, L., Science (1990) 249:505-510; Joyce, G. F., Gene (1989) 82:83-87. Such oligonucleotides have been termed "aptamers" herein. Tuerk and Gold describe the use of a procedure termed "systematic evolution of ligands by exponential enrichment." In this method, a pool of RNAs that are completely randomized at specific positions is subjected to selection 25 for binding by a desired nucleic acid-binding protein which has been fixed on a nitrocellulose filter. The bound RNAs then are recovered and amplified as double-stranded DNA that is competent for subsequent in vitro transcription. The newly transcribed RNA then is recycled through this procedure to enrich for oligonucleotides that have consensus sequences for binding by the cognate protein. The oligonucleotides so obtained then may be sequenced for

further study. Tuerk and Gold applied this procedure to identify RNA oligonucleotides which are bound by the RNA binding region of T4 DNA polymerase.

The identification of oligonucleotides that specifically bind to biomolecules that do not normally bind to RNA or DNA has now been demonstrated for a number of biomolecules that
5 vary widely in size, structure and composition. These molecules include: (1) thrombin, a multifunctional regulatory protein that converts fibrinogen to fibrin in the process of clot formation; (2) bradykinin, a nonapeptide kinin involved in blood pressure regulation and implicated in hypotension; (3) PGF2. α ., a prostaglandin or fatty acid derivative that exhibits hormonal activity. Additionally, the interaction of oligonucleotides with biomolecules whose
10 natural biological function is primarily extracellular has now been demonstrated.

A plasmid is constructed using the methods describe above to include a sequence of interest encoding for an aptamer to thrombin. Aptamers having a sequence of nucleotide bases specifically binding to thrombin are described in U.S. Patent No. 5,840,867. The plasmid is administered to the patient under conditions whereby the plasmid enters cells and generates the
15 aptamer. Alternatively, an ex vivo administration is performed where cells are removed from a patient, the plasmid is transfected into the cells, and the cells are then placed back into the patient. The aptamer binds specifically to thrombin, thereby selectively controlling the biological activity of thrombin, and alleviates the pathological conditions related to thrombin's presence. This plasmid is used either prophylactically or therapeutically.

20 Although described with reference to the figures and specific examples set out herein, those skilled in the art will recognize that certain changes can be made to the specific elements set out herein without changing the manner in which those elements function to achieve their intended respective results. For instance, the cassette described herein is described as being made up of three primary components, genetic elements which comprises a sequence of interest and primer binding site, and a reverse transcriptase gene, each of these components
25 being provided with appropriate promoters as described herein. Those skilled in the art will recognize that, for instance, the MoMuLV reverse transcriptase gene described for use as the reverse transcriptase gene of the cassette can be replaced with other reverse transcriptase genes and that promoters other than the CMV promoter may be used to advantage. All such changes
30 and modifications which do not depart from the spirit of the present invention are intended to fall within the scope of the following non-limiting claims.

Table I: Oligodeoxynucleotides

ODN-PMMV(+) 129 bases (#23)	5' -CTAGGTGGCGGCCGCAAGATTGGTGCACACACACAACGCG CACCAATCTCGCGGCCGCCGACCCGTACAGCGGGGTCTTCATTG GGGGCTCGTCCGGATCGGGAGACCCCTGCCAGGGCC-3'
ODN-PMMV(-) 121 bases (#24)	5' -CTGGGCAGGGGTCTCCGATCCCGACGAGCCCCAAATGAAAG ACCCCCGCTGACGGTCGGCGGCCGAAGATTGGTGCGCGTTGTGT GTGTGCGCACCAATCTCGCGGCCGCCAC-3'
ODN-Test (+) 57 bases (#38)	5' -GGCGGAAGATTGGGGGCCAAAGAGTAACCTCTCAAAGGCACGC GCCCAATCTTCC-3'
ODN-Test (-) 57 bases (#39)	5' -GGCGGAAGATTGGGGCGCGTGCCTTGAGAGTTACTCTTGGC GCCCAATCTTCC-3'
ODN-Telo (+) 92 bases (#40)	5' -GGCGGAAGATTGGGGCGTTAGGGTTAGGGTTAGGGTTAGGGTT AGGGTTAGGGTTAGGGTTAGGGTTAGGGTAGGGCGCCCAATCTTC C-3'
ODN-Telo (-) 92 bases (#41)	5' -GGCGGAAGATTGGGGGCCCTAACCTAACCTAACCTAACCTAAC CTAACCTAACCTAACCTAACCTAACCTAACGCCCAATCTTC C-3'
ODN-XB(+) 51 bases	5' -GGCCTTGAAGAGCGGCCGCACTAACACCACAGTGCGGCCGC TCTTCAA-3'
ODN-XB(-) 51 bases	5' -GGCCTTGAAGAGCGGCCGCACGTGGTGGTGTAGTGCGGCCGC TCTTCAA-3'
ODN-RT (+) 32 bases (#13)	5' -GGGATCAGGAGCTCAGATCATGGGACCAATGG-3'
ODN-RT (-) 24 bases (#12)	5' -CTTGTGCACAAGCTTGCAGGTCT-3'
ODN-N>S (+) 18 bases (#25)	5' -CTAGCGCAAGCGTAGCT-3'
ODN-N>S (-) 10 bases (#26)	5' -ACGCTTGCCTG-3'
ODN-Mbo (+) 30 bases (#16)	5' -CAATTAAGGAAAGCTTGAAGGAAATTATGTC-3'
ODN-Mbo (-) 27 bases (#33)	5' -TAATGGCCCGGGCATAGTCGGTAGGG-3'
ODN-HisPro (+) 43 bases (#36)	5' -AGCTGGATCCCCGCTCCCCACCACCAACACCACCTGCCCT- 3'
ODN-HisPro (-) 42 bases (#37)	5' -AGCAGGGGCAGGGTGGTGGTGGTGGGAGCGGGGGATCC-3' ,
ODN-Rep(+) 121 bases	5' -ATATCTATTAAATTGGCAAATCATAGCGTTATGCTGACTCAG GTGAATGCCCGATAATTTCAGATTGCAATCTTCATCAATGAATT TCAGTGATGAATTGCCAAGATTGATGTGTC-3'
ODN-Rep(-) 111 bases	5' -GACGAGATCTCCTCCAGGAATTCTCGAGAATTGGATCCCCGC TCCCCACCACCAACACCACCAACCCCTGCCCGCGATGAAAAATTATG TGAGCAACATCAATCTTGGC-3'
Seq ID 15	5' -CTAGTCGGATGCGGCCGCTGCACAACACACAACACACAGCGGC CGCATCCGATCAGCGGGGTCTTCATTGGGGCTCGTCCGGATCG GGAGACCCCTGCCAGGGCC-3'
Seq ID 16	5' -CTGGGGCAGGGGTCTCCGATCCGGACGAGCCCCAAATGAAAG ACCCCCGCTGATCGGATGCGGCCGCTGTGTTGTTGTGCA CGGCCGCATCCGA-3'

WHAT IS CLAIMED IS:

1. A set of genetic elements for delivery into a cell comprising:
 - a nucleic acid construct comprising a sequence of interest; and
 - a primer binding site for a reverse transcriptase located in a 3' position with respect to the sequence of interest.
2. A set of genetic elements according to Claim 1, further comprising a reverse transcriptase gene.
3. A set of genetic elements according to Claim 2, wherein the reverse transcriptase gene is polycistrionically transcribable with the sequence of interest and primer binding site.
4. A set of genetic elements according to Claim 2 or 3, wherein the reverse transcriptase gene is located on the same nucleic acid construct as the sequence of interest and primer binding site.
5. A set of genetic elements according to any one of Claims 2 to 4, wherein the reverse transcriptase gene is located in a 5' position with respect to said sequence of interest and 3' primer binding site.
6. A set of genetic elements according to any one of Claims 2 to 4, wherein the reverse transcriptase gene encodes a reverse transcriptase/RNase H polyprotein.
7. A set of genetic elements according to Claim 6, wherein the gene encoding reverse transcriptase/RNase H polyprotein is from Moloney murine leukaemia virus, human immunodeficiency virus, or simian immunodeficiency virus.
8. A set of genetic elements according to any one of Claims 2 to 7, wherein the primer binding site is specific for a reverse transcriptase encoded by the reverse transcriptase gene.
9. A set of genetic elements according to Claim 1, wherein the primer binding site is specific for an endogenous reverse transcriptase.
10. A set of genetic elements according to any one of the preceding claims, wherein the primer binding site is complementary to a transfer RNA (tRNA).
11. A set of genetic elements according to any one of the preceding claims, further comprising a promoter and, optionally, an enhancer for each of said sequence of interest and/or said reverse transcriptase gene.

12. A set of genetic elements according to Claim 11, wherein the promoter and/or enhancer is a eukaryotic promoter and/or enhancer.

13. A set of genetic elements according to Claim 11 or 12, wherein the promoter is a constitutive, inducible, wide-spectrum or tissue specific promoter.

14. A set of genetic elements according to any one of the preceding claims, further comprising a polyadenylation tail sequence located in a 3' position with respect to the sequence of interest and 3' primer binding site.

15. A set of genetic elements according to any one of the preceding claims, wherein the sequence of interest is an antisense sequence.

16. A set of genetic elements according to any one of Claims 1 to 14, wherein the sequence of interest is an aptamer.

17. A set of genetic elements according to any one of the preceding claims, wherein the nucleic acid construct is DNA.

18. A set of genetic elements according to any one of the preceding claims incorporated into at least one vector.

19. A set of genetic elements according to any one of Claims 2-17, wherein the sequence of interest and 3' primer binding site are incorporated into a first vector, and wherein the reverse transcriptase gene is incorporated into a second vector.

20. A set of genetic elements according to any one of Claims 2-17, wherein the reverse transcriptase gene, sequence of interest and primer binding site are incorporated into a single vector.

21. A set of genetic elements according to Claim 20, wherein the reverse transcriptase gene is located in a 5' position with respect to the sequence of interest and 3' primer binding site.

22. A set of genetic elements adapted for delivery into a cell comprising

- (a) a sequence of interest and a 3' primer binding site; and
- (b) a reverse transcriptase gene,

said sequence of interest and 3' primer binding site, and said reverse transcriptase gene being incorporated into at last one vector for delivery into the cell.

23. A vector which comprises:

- (a) a primer binding site and an insertion site for a sequence of interest, the primer binding site being located in a 3' position with respect to the insertion site; and
- (b) a reverse transcriptase gene.

24. A vector according to Claim 23, wherein the reverse transcriptase gene is located in a 5' position with respect to the insertion site and 3' primer binding site

25. A vector system which comprises a first vector, comprising an insertion site for a sequence of interest and a 3' primer binding site and a second vector which comprises a reverse transcriptase gene.

26. A vector or vector system according to any one of Claims 23 to 25, wherein the vector is a plasmid or modified viral construct.

27. A vector or vector system according to any one of Claims 23 to 26, wherein the reverse transcriptase gene is operably linked to an expression control sequence.

28. A host cell stably transformed or transfected with a vector or vector system according to any of Claims 23 to 27.

29. A host cell according to Claim 28, which is a eukaryotic cell.

30. A kit for producing a single-stranded nucleic acid sequence, which kit comprises a vector or vector system according to any one of Claims 23 to 27, and a restriction endonuclease for the insertion site.

31. A kit for producing a single-stranded nucleic acid sequence, which kit comprises a vector or vector system according to any one of Claims 23 to 27, a container for the vector/vector system and instructions for use of the vector/vector system.

32. An *in vivo* or *in vitro* method of producing a single-stranded nucleic acid sequence of interest, which method comprises the steps of introducing a nucleic acid construct into a target cell, the nucleic acid construct comprising a sequence of interest and a primer binding site located in a 3' position with respect to the sequence of interest, transcribing the nucleic acid construct into mRNA and reverse transcribing the mRNA into cDNA.

33. A method according to Claim 32, further comprising the step of removing the mRNA from an mRNA/cDNA heteroduplex formed by reverse transcription of the mRNA.

34. A method according to Claim 32 or 33, wherein reverse transcription is carried out by a reverse transcriptase expressed by a reverse transcriptase gene introduced into the target cell.

35. A method according to Claim 32 or 33, wherein reverse transcription is carried out by a reverse transcriptase which is endogenous to the target cell.

36. A method according to any one of Claims 33 to 35, wherein the mRNA transcript is removed from the mRNA/cDNA heteroduplex by means of RNase H.

37. A method according to Claim 36, wherein the RNase H is expressed from a gene encoding a reverse transcriptase/RNase H polyprotein introduced into the target cell.

38. A method according to any one of Claims 32 to 37, further comprising the step of isolating the mRNA transcript, mRNA/cDNA heteroduplex and/or single-stranded cDNA from the target cell.

39. A single-stranded cDNA transcript produced by the method of any one of Claims 32 to 38.

40. An inhibitory nucleic acid molecule produced by the method of any one of Claims 32 to 38.

41. An inhibitory nucleic acid molecule according to Claim 40, which is an antisense sequence or an aptamer.

42. An mRNA transcript produced by the method of any one of Claims 32 to 38.

43. A heteroduplex molecule produced by the method of any one of Claims 32 to 38.

44. A pharmaceutical composition which comprises a set of genetic elements according to any one of Claims 1 to 22, together with a pharmacologically acceptable adjuvant, diluent or carrier.

45. A pharmaceutical composition which comprises a vector or vector system according to any one of Claims 23 to 27, together with a pharmacologically acceptable adjuvant, diluent or carrier.

46. A pharmaceutical composition which comprises a host cell according to Claim 28 or 29, together with a pharmacologically acceptable adjuvant, diluent or carrier.

47. A set of genetic elements according to any one of Claims 1 to 22 for use in therapy, especially for use in delivering an inhibitory nucleic acid molecule to a target cell.

48. A vector or vector system according to any one of Claims 23 to 27 for use in therapy, especially for use in delivering an inhibitory nucleic acid molecule to a target cell.

49. A host cell according to Claim 28 or 29 for use in therapy, especially for use in delivering an inhibitory nucleic acid molecule to a target cell.

50. Use of a set of genetic elements according to any one of Claims 1 to 22, for the manufacture of a medicament for alleviating a pathological condition by regulating gene expression, especially for alleviating a pathological condition by delivery of an inhibitory nucleic acid molecule to a target cell.

51. Use of a vector or vector system according to any one of Claims 23 to 27, for the manufacture of a medicament for alleviating a pathological condition by regulating gene expression, especially for alleviating a pathological condition by delivery of an inhibitory nucleic acid molecule to a target cell.

52. Use of a host cell according to Claim 28 or 29 for the manufacture of a medicament for alleviating a pathological condition by regulating gene expression, especially for alleviating a pathological condition by delivery of an inhibitory nucleic acid molecule to a target cell.

1/3

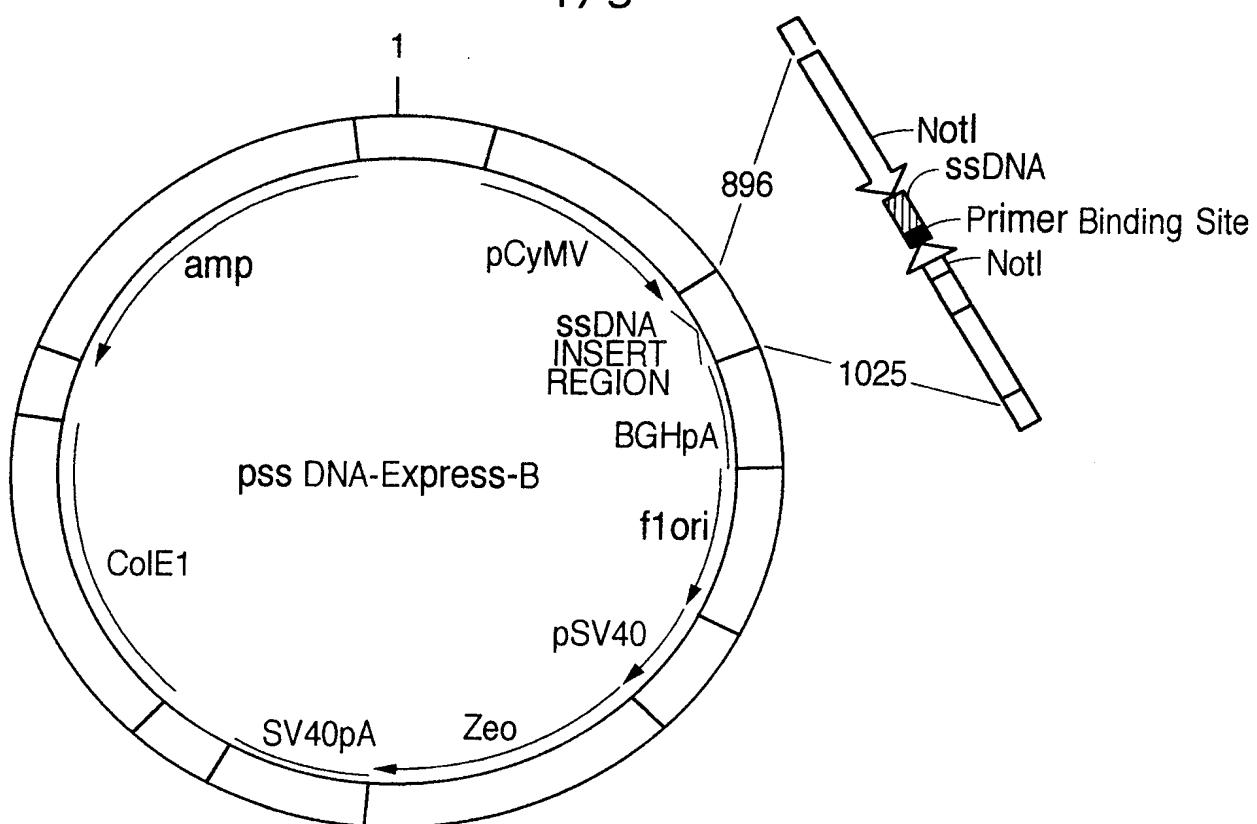


Fig. 1A

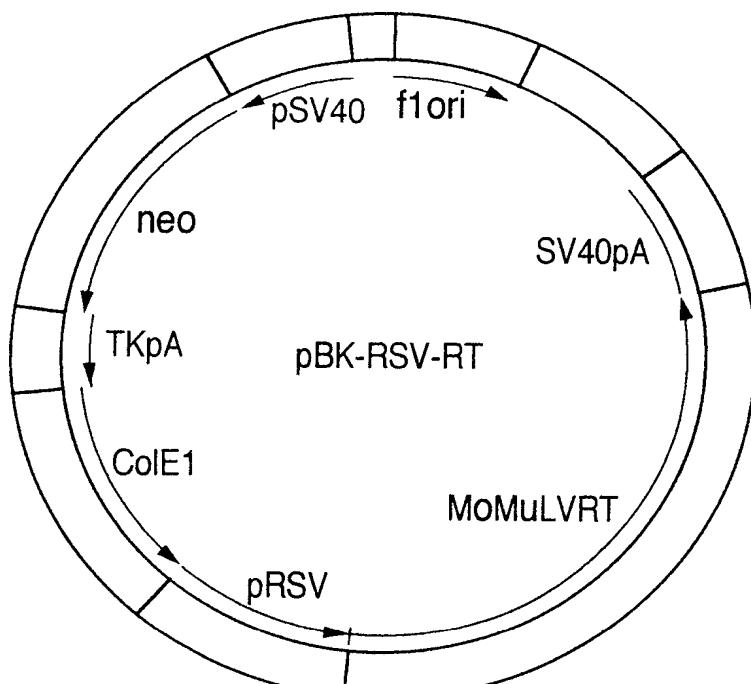


Fig. 1B

2/3

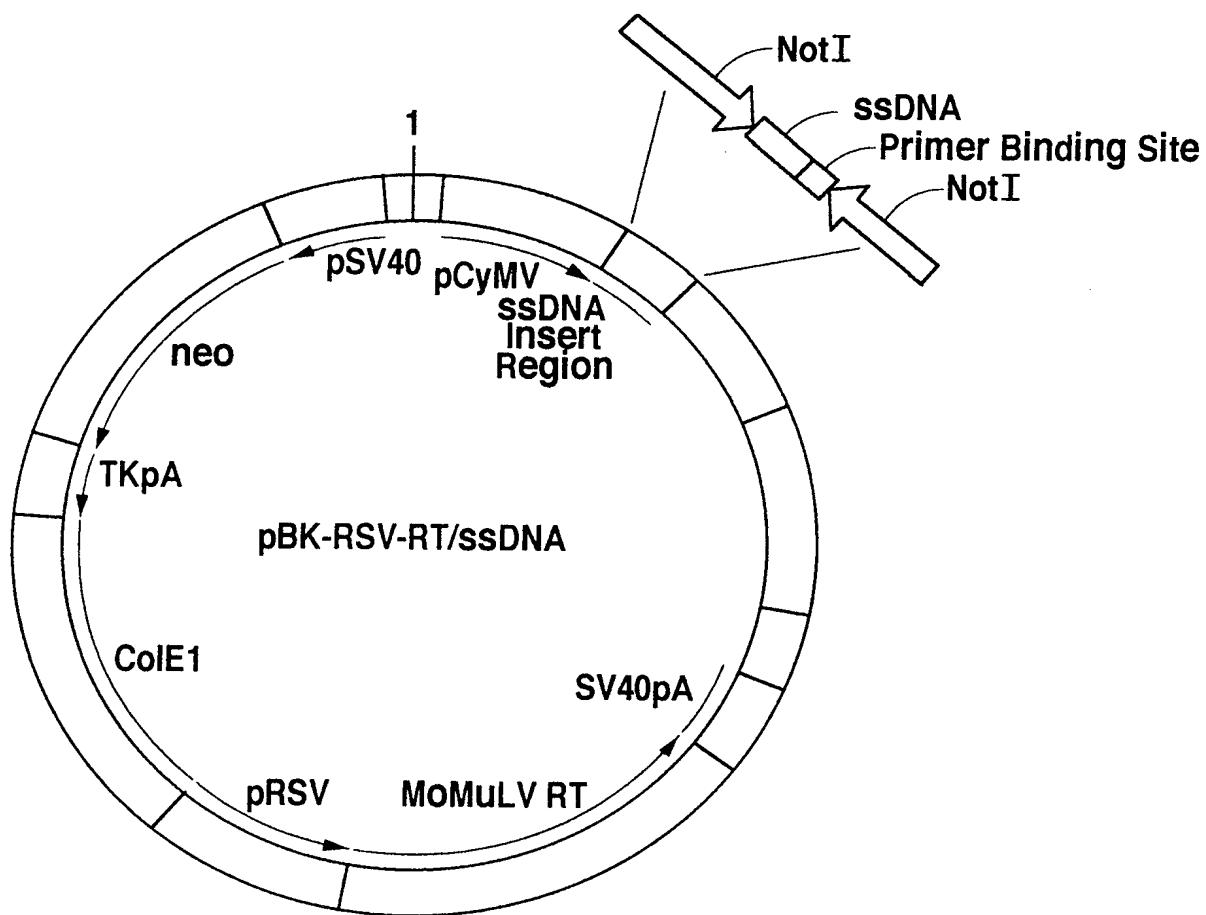


Fig. 1C

3/3

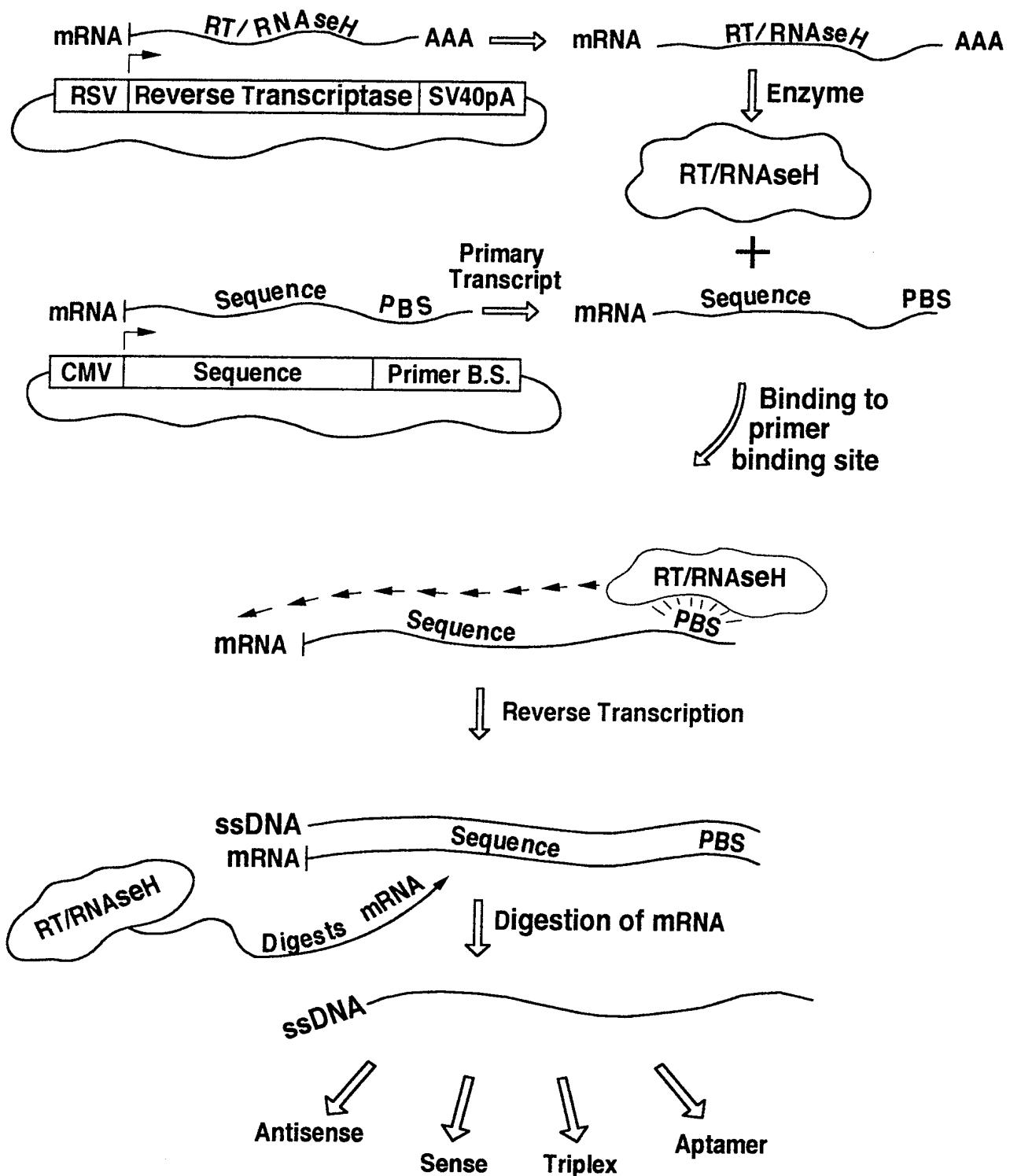


Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/23933

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7	C12N15/10	C12N15/11	C12N15/85	C12N5/10	A61K31/711
					A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

Date of mailing of the international search report

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INTERNATIONAL SEARCH REPORT

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